Attorney's Docket No.:

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Art Unit	:
Serial No.	: 09/889,867	Examiner	:
Filed	:		
Title	: Chaperonin 10 and beta-interferon therapy of multiple sclerosis		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

Sir:

1. I, Barbara Johnson, Ph.D., am Head of Immunology at CBio Limited, Queensland, Australia, the exclusive licensee of the above-identified patent application.

2. I am an expert in the field of immunology and in the field of conducting and managing clinical trials for the development of therapies for numerous diseases. I have been active in biotechnology/immunology research for more than 20 years, having most recently taken a lead protein through preclinical studies and safely through phase I and early phase II clinical trials. My resume is attached as documentation of my credentials.

3. I have read the specification and the file history, including past and the outstanding office actions, and Applicants' responses, for the above-referenced patent application U.S. Serial No. 09/889,867 (hereinafter referred to as "the application"), and I understand the issues presented by the Patent Office in the outstanding office action regarding the pending claims of the application (hereinafter "the invention").

4. I believe that there was a long-felt need for an invention such as that set forth in the pending claims. Multiple sclerosis (MS) is a debilitating auto-immune disease affecting millions of individuals worldwide. Throughout the 20th century scientists and physicians have sought effective treatments for MS, with limited success.

5. I believe the invention offers a solution to this long felt need by providing an effective therapy combining beta-interferon and chaperonin 10. It is worthy to note that in spite of years of intensive research by many investigators no one had, before the present invention, taught or suggested a therapy for MS comprising combined administration of beta-interferon and chaperonin 10.

6. Further, it is my opinion that, prior to the invention there was no suggestion in the art that combining beta-interferon and chaperonin 10 (otherwise known as Early Pregnancy Factor or EPF) would provide a beneficial effect. Indeed it is not possible to predict in advance the outcome of combining two agents, whether or not these agents have been shown to have an effect independently. This is particularly true with autoimmune diseases such as MS. The result may be deleterious, of no benefit or of some benefit.

7. In this regard, in a paper by Douglas R. Jeffery, MD, PhD entitled "Use of combination therapy with immunomodulators and immunosuppressants in treating multiple sclerosis" (Neurology 63 (Suppl 6):S41-S46, 2004; copy attached), the author states that a drawback of using combination therapy for treating MS is that "the agent added to the primary therapy may have no effect, or, worse, may antagonize the effect of the primary agent." The author goes on to warn (page S42, first column) that in many instances whilst one may assume that the combination of two agents may have a beneficial effect, the opposite may be true and "[F]or example, agents that should, in theory, improve the effect of [IFNβs or GA] might instead block their effects" such that "in the absence of data suggesting that a particular combination of agents is more effective than either one alone, that combination should be avoided". "One cannot assume, on the basis of mechanism of action, that the addition of an agent to a standard IMA is safe. The new drug may have no effect or may even antagonize the effect of the [other agent]." This author goes on to site examples of drugs which when used alone in the treatment of disease have been shown to be beneficial, yet when used in combination therapy they have in fact worsened the symptoms and progression of MS.

8. The uncertainty over the effect of combination therapy is exacerbated where there is a lack of understanding or knowledge of the mechanism of action of one or both of the agents. In fact, this was the case for beta-interferon and chaperonin 10 at the time of the invention. However evidence at the time led to the prevailing view prior to the invention which was that these agents acted via the same or similar immunosuppressive mechanisms. The article by Morton, H., 1998 (*Immunol Cell Biol* 76:483-496; copy attached) states that EPF acted to suppress antigen- or mitogen-stimulated T cell proliferation *in vitro* and the DTH reaction *in vivo*. Likewise a paper published by Yu et al., 1996 (*J Neuroimmunol* 64:91-100; copy attached) describes data indicating that IFNβ inhibits myelin antigen-stimulated T cell proliferation *in vitro* and limits the antigen-induced DTH reaction *in vivo*. In view of these similarities it was reasonable to assume that beta-interferon and chaperonin 10 acted via similar immunosuppressive mechanisms.

9. If beta-interferon and chaperonin 10 did indeed act by similar mechanisms, a practitioner in the field would not expect that their combination would have any co-operative effect. Thus, there would be no motivation to combine these similarly acting agents. Indeed, there was no suggestion in the art to do so prior to the invention. In fact, the prior art implicitly taught away from the present invention by teaching that beta-interferon and chaperonin 10 have the same biological mechanism of action.

10. The inventors of the invention were the first to realize that beta-interferon and chaperonin 10 actually act via different mechanisms. It was only with this finding that the possibility of combination therapy using beta-interferon and chaperonin 10 became feasible. By discovering that beta-interferon and chaperonin 10 act via different mechanisms, the inventors of this invention were the first to use combination therapy using beta-interferon and chaperonin 10.

11. However, even in the knowledge that beta-interferon and chaperonin 10 have differing mechanisms of action (which was not known prior to the invention) it does not follow that it is predictable that the two agents when combined will have a co-operative effect.

There are numerous examples where in attempting to improve treatment for a variety of diseases, two agents having been shown to be efficacious independently do not have any beneficial effect in combination. For example, a 2003 press release from the National Institute of Arthritis and Musculoskeletal and Skin Diseases reported the results of a study that combining parathyroid hormone with alendronate produces "no significant improvement in bone mineral density beyond that produced by the individual drugs". Further, also in 2003 the results of a study into obesity treatment concluded that combining sibutramine and orlistat, each exhibiting substantial effects on weight individually, did not offer any benefit over single agent therapy.

12. For the reasons discussed above, it is my expert opinion, and I believe it would be the opinion of other experts in the field that prior to the present invention it would not have been obvious to combine beta-interferon and chaperonin 10 for the treatment of MS.

13. In my opinion it is clear from the results provided in the present patent application that the administration of beta-interferon and chaperonin 10 in combination do provide a beneficial and synergistic effect over the administration of each alone and further that this is achieved using doses of each agent lower than would be regarded as optimal for each agent used alone. Table 4 directly compares the cumulative disability score in EAE mice following administration of 2.5 μg Cpn10/mouse/every 2nd day and 5,000 IU IFNβ/mouse/every 2nd day, either administered alone or together. The data in Table 3 demonstrate a dose-responsive reduction in cumulative disability score when chaperonin 10 is administered at doses ranging from 2.5 - 10 μg/mouse/every 2nd day. Further it is noted that in the prior art referred to by the Examiner, Morton et al. (WO 95/15338), the dose of chaperonin 10 administered to EAE rats was 15 μg. Thus 2.5 μg can be considered a suboptimal dose. Similarly, Yu et al (discussed above) demonstrate a dose-response curve of IFNβ between 5,000 and 10,000 IU in the treatment of EAE induced by PLP (the same system as employed by Morton et al). Therefore, 5000 IU of IFNβ can also be considered a suboptimal dose in this model.

14. Using this combined treatment regime, that is both agents used at suboptimal doses, in EAE mice, a synergistic effect on disease score at both the primary attack timepoint (d8-21) and during the relapse (at d22-60) was observed as shown in Table 4 (respective p-values of 0.039 and 0.014 clearly demonstrating the statistical significance of these results). The fact that both agents were believed to be acting via the same or similar mode of action is crucial in the description of the combined effects of these drugs having a synergistic effect. As noted above, this would not have been predicted, nor was it contemplated, prior to the invention.

I hereby declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully Submitted

Date: 27 October 2005

B. Johnson

Barbara Johnson, PhD

CURRICULUM VITAE

Barbara J. Johnson
10 Villosa St
Shailer Park, QLD 4128
07 3209 9950 or 0407 448843
email: barbara.johnson@cbio.com.au

EDUCATION:

2002 Postgraduate Studies Programme in Drug Development, University of New South Wales, NSW.

1992 Ph.D., Veterinary Microbiology, Texas A&M University, College Station, TX

1985 M Sc., Medical Parasitology, London School of Hygiene and Tropical Medicine, University of London, UK

1984 Single Subject Teaching Credential in Life Sciences, University of California, Santa Barbara, CA

1983 B.A., Zoology, University of California, Santa Barbara, CA

PROFESSIONAL EXPERIENCE:

2002-present **Head of Immunology, R&D Department**, CBio Ltd, Brisbane, Qld.

2001-2002 **Research Officer**, Department of Medicine, University of Queensland and The Mater Adult Hospital, Brisbane, QLD

2000-2001 **Writer**, Santa Barbara Community College, Santa Barbara, CA

1997-2000 **Research Officer**, Leukocyte Biology Unit, Queensland Institute of Medical Research, Brisbane, QLD, Australia

1994-1997 **Research Associate**, Laboratory of Cellular Physiology & Immunology, The Rockefeller University, New York, NY

1992-1994 **Post-doctoral Associate**, Laboratory of Cellular Physiology & Immunology, The Rockefeller University, New York, NY

1988-1992 **Graduate Assistant**, Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX

1987-1988 **Senior Technologist**, Disease Detection International and Environmental Diagnostics Inc., Burlington, NC

1986-1987 **Teacher**, Big Pine Unified School District, CA

1985-1986 **Junior Investigator**, Ministry of Health, Government of Sudan

RESEARCH ACTIVITIES:

2002 – present During the past four years I have organized and managed the pre-clinical and phase I clinical trial programmes at CBio Ltd. Working with this start-up biotechnology company, I have been responsible for designing and implementing the pre-clinical package, including laboratory assays for drug potency, detection of anti-drug antibodies, and release assays. I designed the animal toxicology programme and managed this and other out-sourced research-based studies towards development of a pre-clinical safety data package. Leading up to our phase I clinical trials, I wrote the Investigator's Brochure, designed and co-wrote the trial protocol, and put together the submission for clinical trial to the relevant Human Ethics Review Boards.

During two phase I clinical trials, I supervised the trials on-site, served as the company's clinical trial monitor and performed 100% Source Data Verification on each trial volunteer. I also trained and supervised others in the performance of procedures in blood processing, storage and shipment from the laboratory at the clinical trial site to our laboratory at CBio Ltd. I was then in charge of running laboratory assays on those stored blood products and reporting laboratory results in reports of drug performance, including pharmacokinetics, pharmacodynamics and other safety reports. These became part of the clinical trial reports and I ultimately incorporated them into the updated Investigator's Brochure in preparation for our phase II clinical trial submissions.

In the past year my focus has shifted from clinical trials to running the Immunology section of the Research and Development Department within CBio, managing a team of scientists in exploring and reporting on the mechanism of action of compounds of interest to the company.

1997-2000 Investigation of the role of perforin-mediated $CD8^+$ T cell-directed cytotoxicity during murine pulmonary influenza infection. Analysis of how perforin and the granzymes cooperate during lytic activity, and function of the granzymes in resolution of viral infection. This project was funded by the CRC for Vaccine Technology.

1992-1997 Analysis of the role of cytokines during the course of tuberculosis infection, and their potential modulation to enhance the antimicrobial immune response. Conducted four clinical studies in Bangladesh and in Cape Town, South Africa in which patients with drug-sensitive or multidrug-resistant tuberculosis were treated with recombinant human interleukin-2 in combination with multidrug therapy. Following each clinical study, patient sera and RNA extracted from tissues and peripheral blood cells were analyzed for levels of cytokines and cytokine mRNA expression at various time points during the studies. Patient RNA samples were then subjected to differential display analysis of IL-2 induced changes in gene expression.

1988-1992 Analysis of the coordination and regulation of the leukocyte response to *Mycobacterium tuberculosis*-infected macrophages using flow microfluorimetry to determine changes in T cell subpopulations and quantitative RT-PCR technology to analyze cytokine gene expression at the mRNA level.

1987-1988 Development of solid phase immunodiagnostic assays for human parasitic diseases, for drugs of abuse, and for mycotoxins.

1985-1986 Investigation of the immune response of individuals hyper-infected with *Schistosoma mansoni* in the Nile delta region of Sudan. This project was funded by USAID and supervised by investigators at Michigan State University, MI.

1984-1985 Isolation, purification, and characterization of tegument antigens of a larval stage of the helminth parasite, *Schistosoma mansoni*.

PUBLICATIONS:

Johnson, Barbara J. and David McMurray. Cytokine gene expression by cultures of human lymphocytes with autologous *Mycobacterium tuberculosis*-infected monocytes. Infection and Immunity. 62:1444-1450, 1994.

Tramontana, Jane M., Utaiwan Utaipat, Anthony Molloy, Pasakorn Akarasewi, Margaret Burroughs, Sanit Makonkawkeyoon, **Barbara Johnson**, Jeffrey Klausner, William Rom and Gilla Kaplan. Thalidomide treatment reduces TNF-α production and enhances weight gain in patients with pulmonary tuberculosis. Molecular Med. 1:384-397, 1995.

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Moreira, Andre L., Laura G. Corral, Weiguo Ye, **Barbara Johnson**, David Stirling, George W. Muller, Victoria H. Freedman, and Gilla Kaplan. Thalidomide and thalidomide analogs reduce HIV type 1 replication in human macrophages *in vitro*. AIDS Research and Human Retroviruses. 13:857-863, 1997.

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Doyle, Anthony G., Kathy Buttigieg, Penny Groves, **Barbara J. Johnson**, and Anne Kelso. The activated type 1-polarized $CD8^+$ T cell population isolated from an effector site contains cells with flexible cytokine profiles. Journal of Experimental Medicine 190:1081-1091, 1999.

Kelso, A, Costelloe, EO, **Johnson BJ**, Groves P, Buttigieg K, Fitzpatrick DR. The genes for perforin, granzymes A-C and IFN-γ are differentially expressed in single $CD8^+$ T cells during primary activation. International Immunology 14(6) 605-613, 2002.

Johnson, Barbara J., Elaine O. Costelloe, David R. Fitzpatrick, John B.A.G. Haanen, Ton N.M. Schumacher, Lorena E. Brown, and Anne Kelso. Anatomical differences in perforin and granzyme expression patterns of $CD8^+$ T cells in influenza virus-infected mice. Journal of Virology. Submitted.

Johnson, Barbara J. (cont.)

Other Publications

I wrote the text for an internet-based Molecular Biology tutorial for second year Life Science students enrolled at Santa Barbara Community College.

Technical Publications

Johnson, Barbara. 1991. Cell to cell contact across transwell membranes. Technical Bulletin, Becton Dickinson Labware.

Theses

Johnson, Barbara. 1992. The coordination of the leukocyte response to *Mycobacterium tuberculosis*-infected macrophages. Ph.D. Dissertation, Texas A&M University, TX.

Tanner, Barbara Johnson. 1985. The solubilization and purification of a Mr 16kD *Schistosoma mansoni* schistosomular surface antigen recognized by McAb M7B3A. Masters Thesis, London School of Hygiene and Tropical Medicine, University of London.

Selected Conference Presentations

Alteration of phenotype distribution in human lymphocytes cultured with monocyte/macrophages infected with virulent *Mycobacterium tuberculosis*. American Society for Microbiology, New Orleans, LA. May 1992.

Cytokine expression induced by coculture of human T cells with macrophages infected with virulent *Mycobacterium tuberculosis* H37Rv. FASEB, Los Angeles, CA. May 1992.

Clinical and immune responses of TB patients treated with low dose IL-2. Keystone Symposia on Molecular and Cellular Biology: Molecular Mechanisms in Tuberculosis. Tamarron, CO Feb 1995.

Cytokines and other inflammatory mediators in the immune response to tuberculosis.

Invited Speaker. American Society for Microbiology, New Orleans, LA. May 1996.

Use of differential display RT-PCR to analyze changes in gene expression in multidrug resistant tuberculosis patients following treatment with rhuIL-2. Differential Display and Related Techniques for Gene Discovery, Cold Spring Harbor Laboratory, NY. Oct 1996.

rhuIL-2 adjunctive therapy in multidrug-resistant tuberculosis: A comparison of two treatment regimes. Invited speaker. Thirty-second U.S.-Japan Cooperative Medical Science Program. Tuberculosis-Leprosy Research Conference. July 1997.

Perforin- and granzyme-mediated $CD8^+$ T cell directed cytoxicity during murine influenza virus infection. Keystone Symposia: Determinants of Immune Defense against Microbial Infections. Santa Fe, NM Feb 1999.

Professional References

Anne Kelso, Professor
Relationship: Previous supervisor
Leukocyte Biology Unit
Queensland Institute of Medical Research
Brisbane, QLD
Tele: 07 3362.0382

Michael Good, Director
Relationship: Previous supervisor on CRC-VT-funded project
Queensland Institute of Medical Research
Brisbane, QLD
Tele: 07 3362.0202

Gilla Kaplan, Associate Professor
Relationship: Previous Supervisor
Laboratory of Cellular Physiology & Immunology
Rockefeller University
Box 176; 1230 York Ave
New York, NY 10021
Fax: 212.327.8875
Tele: 212.327.8375

Michel Rabinovitch, Professor
Relationship: Colleague and Collaborator
Universidade Federal de Sao Paulo
Escola Paulista de Medicina
Disciplina de Parasitologia
Rua Botucatu 862, 60 adnar
04023-062 Sao Paulo, SP Brazil
Fax: 55.11.571.10.95
E-mail: rabinom.dmip@epm.br

David McMurray, Professor
Relationship: Ph.D. Advisor
Department of Medical Microbiology & Immunology
College of Medicine
Texas A&M University
College Station, TX 77843-1114
409.845.1367

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Journal of Neuroimmunology 64 (1996) 91–100

Journal of Neuroimmunology

Interferon-β inhibits progression of relapsing–remitting experimental autoimmune encephalomyelitis

Min Yu [a], Akiko Nishiyama [b], Bruce D. Trapp [b], Vincent K. Tuohy [a,*]

[a] *Department of Immunology, FFb-1, Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, USA*
[b] *Department of Neurosciences, Research Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA*

Received 14 August 1995; revised 18 October 1995; accepted 18 October 1995

Abstract

The results of two phase III clinical trials have recently shown that interferon-beta (IFNβ) is effective in the treatment of relapsing–remitting multiple sclerosis (RRMS). Treatment with IFNβ results in a significant decrease in the rate of clinical relapse and a marked delay in progression to disability compared to placebo-treated control patients. In the present study, we demonstrate similar therapeutic effects after treating (SWR × SJL)F_1 mice with IFNβ at the onset of clinical signs of experimental autoimmune encephalomyelitis (EAE), a disease animal model widely used in MS studies. EAE was actively induced by immunization of (SWR × SJL)F_1 mice with the immunodominant encephalitogenic peptide 139–151 of myelin proteolipid protein (PLP). In blinded testing, mice treated with IFNβ at EAE onset showed a delay in progression to clinical disability as determined by marked improvement with time in mean clinical score, significant delay in onset of relapse, and significant decrease in exacerbation frequency compared to placebo-treated control mice. The therapeutic effect of IFNβ was accompanied by a significant inhibition of delayed-type hypersensitivity (DTH) but not proliferation in response to the priming PLP 139–151. In addition, IFNβ treatment resulted in an overall decrease in severity of both inflammation and demyelination in the central nervous system. These results mimic in an autoimmune animal model the effectiveness of IFNβ treatment observed in MS. Moreover, our study suggests that anti-viral properties of IFNβ are not essential for producing therapeutic effects in autoimmune demyelinating disease, and that the efficacy of IFNβ in the treatment of MS may be due to inhibition of autoreactivity.

Keywords: Interferon-β; Myelin proteolipid protein; Experimental autoimmune encephalomyelitis; Demyelination; Multiple sclerosis; Cytokine treatment

1. Introduction

Although the etiology of multiple sclerosis (MS) is uncertain, a number of studies indicate that MS is an autoimmune demyelinating disease of the central nervous system (CNS) (Lisak, 1980; McFarlin and McFarland, 1982; Weiner and Hauser, 1982; Ellison et al., 1984; Waksman, 1985). In addition, results of epidemiological studies suggest that an environmental agent, presumably a virus, is involved in the etiopathogenesis of MS (Allen and Brankin, 1993; Kurtzke, 1993; Sarchielli et al., 1993). Numerous viruses have been associated with MS (Adams and Imagawa, 1962; Anderson, 1988; Norrby et al., 1994). However, despite extensive attempts at isolating viruses from MS tissue, no consistent pattern has emerged which implicates specific viruses in disease pathogenesis.

Recent clinical trials have shown that interferon-β (IFNβ) is therapeutic in the treatment of patients with relapsing–remitting MS (RRMS). Recombinant, non-glycosylated, human IFNβ-1b (Betaseron™) has been approved for treatment of RRMS patients as a result of a multicenter randomized double-blind placebo-controlled clinical trial which showed that s.c. injection of RRMS patients every other day with Betaseron™ produced a significant decrease in the rate of clinical exacerbation and in disease activity measured by MRI assessment of active and new lesions (Interferon-β Multiple Sclerosis Study Group, 1993; Paty and Li, 1993). More recently, the results of another phase III trial using recombinant, glycosylated, human IFNβ-1a (Biogen®) administered i.m. weekly have confirmed the therapeutic effect of IFNβ in reducing exacerbation rate and MRI activity and have shown a 40% decrease in 1 and 2 year disability progression rates (Jacobs et al., 1994).

Despite confirmed efficacy in MS, the basis for the

* Corresponding author. Phone (216) 445 9684; Fax (216) 444 8372; E-mail tuohyv@cesmtp.ccf.org.

SSDI 0165-5728(95)00160-3

pertussis, the inguinal and axillary LNC were cultured in triplicate test and control wells at 3×10^5 LNC/well in a total volume of 200 μl in 96-well flat-bottomed microtiter Falcon plates (Becton Dickinson, Lincoln Park, NJ). Test cultures contained 20 μM p139–151 and varying concentrations (10–10000 IU/ml) of IFNβ all added simultaneously at the initiation of culture in DMEM (GIBCO BRL) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 2 mM fresh L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 30 mM HEPES buffer, 0.5 mM sodium pyruvate (GIBCO BRL) and 5×10^{-5} M 2-mercaptoethanol. In addition, spleen cells from control and IFNβ-treated mice were tested at 56 days for responsiveness to various doses of p139–151. Triplicate positive control wells contained 10 μg/ml of anti-mouse CD3 (Pharmingen, San Diego, CA) or 0.005 tuberculin units/ml of tuberculin purified protein derivative (PPD, Connaught, Willowdale, Ontario, Canada) while negative control wells contained no peptide. After 48 h of culture, wells were pulsed with [methyl-^{3}H]thymidine (1.0 μCi/well, sp. act. 6.7 Ci/mmol, New England Nuclear, Boston, MA), and the cells were harvested after 16 h by aspiration onto glass fiber filters. Levels of incorporated radioactivity were determined by scintillation spectrometry. Results are expressed as mean counts per minute (cpm) of triplicate experimental cultures with Ag divided by mean cpm of cultures without Ag (stimulation index).

2.7. Delayed-type hypersensitivity (DTH) ear swelling

Determinant-specific DTH reactions were quantitated as previously described (McRae et al., 1992). At 2 and 4 weeks after EAE onset, IFNβ- and PBS-treated (SWR × SJL)F_1 mice were challenged by injecting 10 μl PBS containing 10 μg of p139–151 in the dorsal surface of the ear using a 100 μl Hamilton syringe and a 30 gauge needle. Contralateral ears were injected similarly with PBS containing 10 μg of BSA (Sigma). Ear thickness was measured three times prior to the injection and three more times 24 h after challenge using a Mitutoyo model 7326 engineers micrometer (Schlesinger's Tools, Brooklyn, NY). Increases in p139–151-specific ear swelling were determined and expressed in units of 10^{-4} inches ± S.E.

2.8. Histopathology

(SWR × SJL)F_1 mice were anesthetized and perfused through the heart with 4% paraformaldehyde in 0.08 M Sorenson's phosphate buffer. Brain and spinal cord tissue were removed and placed in fixative overnight. Segments of tissue were cryoprotected in 30% sucrose and frozen on a sliding microtome. For histological analysis 10 μm sections were cut on a cryostat and stained with Luxol fast blue followed by counterstaining with hematoxylin and eosin. At least 9 transverse sections of the spinal cord ranging from cervical to sacral regions were examined and graded for the appearance of cellular infiltrates and demyelination by a person blinded to treatment protocols. Sections from cerebrum and cerebellum were similarly examined. The extent of inflammation was graded as follows: −, no inflammation; ±, few inflammatory cells; +, slightly increased numbers of inflammatory cells and/or the appearance of perivascular cuffs or clusters in the subarachnoid space; + +, moderate inflammatory cell infiltration in the parenchyma; + + +, extensive parenchymal infiltration; + + + +, severe parenchymal infiltration covering almost an entire white matter column. The extent of demyelination was scored as follows: −, no demyelination; ±, few patchy areas of demyelination in otherwise normally appearing myelin; +, small areas of demyelination mostly confined to subpial regions; + +, large areas of demyelination occupying less than one-half of the area of a white matter column; + + +, larger demyelinated areas occupying more than one-half of the area of a white matter column; + + + +, demyelination of an entire white matter column. In all samples, cellular infiltrates were determined by hematoxylin and eosin staining while demyelination was determined by lack of luxol fast blue staining.

2.9. Immunocytochemistry

For immunocytochemical analysis, free-floating sections of fixed cryoprotected CNS tissue were cut (30 μm thick) and immunostained with PLP antibodies using the avidin–biotin complex (ABC) procedure (Vectastain Elite ABC kits, Vector, Burlingame, CA). Sections were pretreated with 1% H_2O_2 in 10% Triton X-100 for 30 min and incubated sequentially with 3% normal goat serum (Sternberger Monoclonals, Baltimore, MD) for 30 min at 22°C, PLP monoclonal IgG2a antibody (Harlan, Indianapolis, IN) diluted 1:200 for 14 h at 4°C, biotinylated goat anti-mouse IgG (Vector) diluted 1:500 for 30 min at 22°C, avidin–peroxidase–biotin complex diluted 1:1000 for 1 h, diaminobenzidine and 0.01% H_2O_2 for 8 min, and 0.4% OsO_4 for 30 s. The sections were then washed in PBS, infiltrated with glycerol, placed on glass slides, and photographed using an Axiophot photomicroscope (Carl Zeiss, Germany).

2.10. Statistical analysis

The Mantel-Haenszel log-rank test was used to detect event-free differences between the treatment groups. In addition, the two-sided Wilcoxon rank sum test was used in analysis of relapses occurring 2–8 weeks after EAE onset. The student t-Test was used to analyze differences in p139–151 DTH and proliferation between IFNβ and PBS treatment groups.

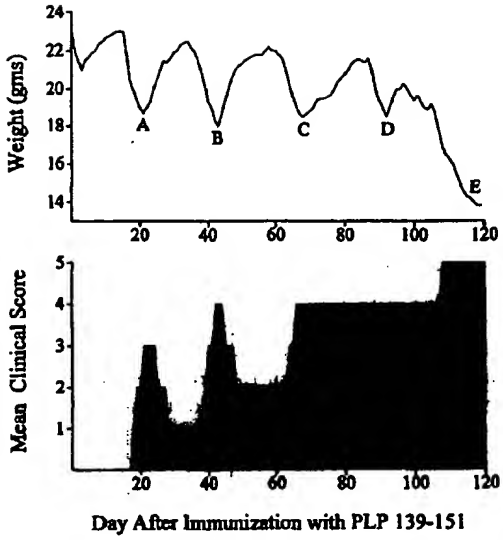

Fig. 1. Clinical course of disease in a representative (SWR × SJL)F_1 mouse immunized with PLP 139–151. Abrupt and substantial (≥ 7%) weight losses (upper graph, A–E) accompany EAE onset (A), two distinct clinical relapses (B and C), and progression to chronic disease (E). The abrupt weight loss occurring around 90 days (D) is not accompanied by an observable increase in clinical score perhaps because of the incremental accumulation of neurological deficit accompanying each incomplete recovery.

relapse 22.5 vs 33.5 days, $P = 0.03$ by the logrank test) and more frequently ($P = 0.01$ by the Wilcoxon Rank Sum test) than SJL/J mice.

3.2. *(SWR × SJL)F_1 mice develop chronic-progressive autoimmune demyelination*

The exacerbating phase of EAE in (SWR × SJL)F_1 mice was routinely followed by progression to chronic disease (Fig. 1). (SWR × SJL)F_1 mice that survived the primary attack of EAE underwent an incomplete recovery within 14 days after onset. All mice routinely retained some residual neuroparalytic signs and invariably relapsed, usually within 30 days of the initial attack. The first relapse was consistently more severe than the initial clinical signs, and the second recovery left mice more impaired than the first. Multiple exacerbations were often observed with each relapse being progressively more severe and with each remission leaving mice progressively more impaired. Substantial decreases and increases of weight were typically associated with exacerbations and remissions, respectively. As the disease progressed, it became increasingly more difficult to observe relapse since each recovery was incrementally more incomplete. By 120 days after EAE onset, the majority of (SWR × SJL)F_1 mice reached a chronic-progressive stage of EAE characterized clinically by gradual inability to initiate self-directed movement, loss of grooming behavior, and up to a 50% reduction of initial body weight. If not meticulously cared for, mice developed skin and mucosal infections and quickly died.

Histological analysis of the CNS revealed perivascular inflammatory foci predominantly in the cervical and lumbar regions of the spinal cord. During the early EAE onset/recovery period, spinal cord lesions typically showed infiltration of inflammatory cells with little evidence of

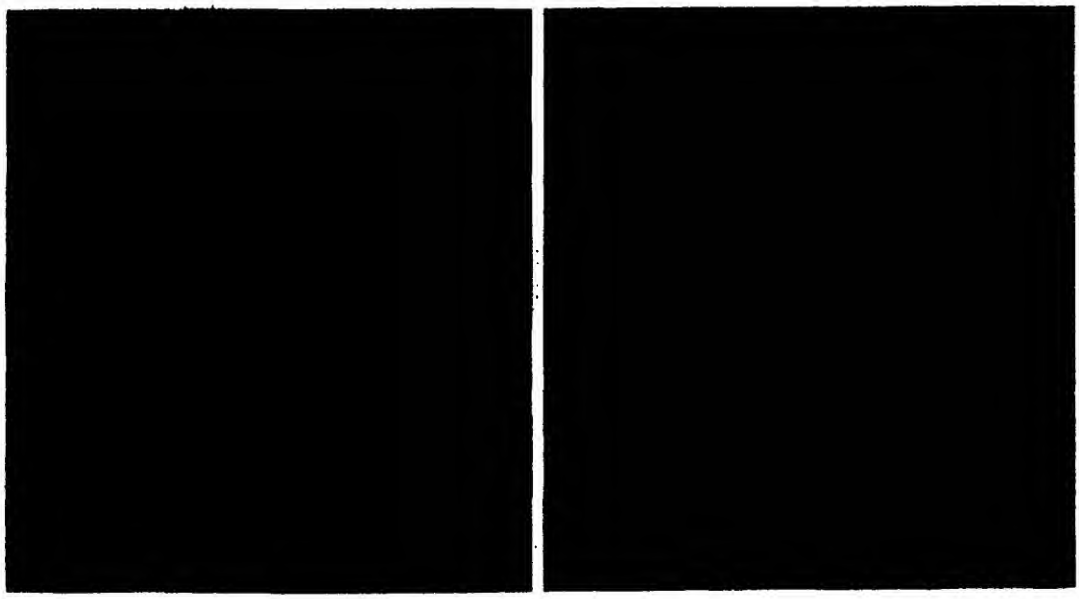

Fig. 2. Immunocytochemical distribution of PLP in cervical spinal cord from control (A) and EAE (B) (SWR × SJL)F_1 mice (magnification 380 ×). EAE mouse was killed 130 days after immunization with the encephalitogenic determinant PLP 139–151. In contrast to the control mouse which is myelinated normally, the mouse with chronic-progressive EAE has extensive demyelination of the dorsal columns. Scale bar represents 50 μm.

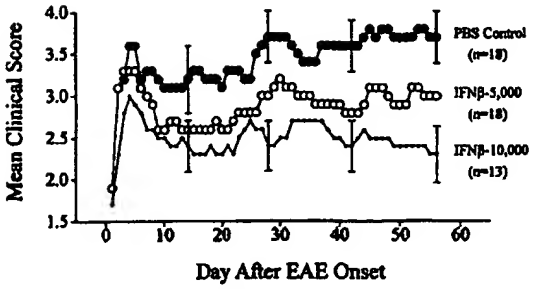

Fig. 3. Mean clinical score (±S.E.) after treatment of (SWR×SJL)F_1 EAE mice with IFNβ or PBS. Includes mice treated with either PBS, 5000 IU IFNβ, or 10000 IU IFNβ injected s.c. every other day from EAE onset to day 56 as described in Materials and methods.

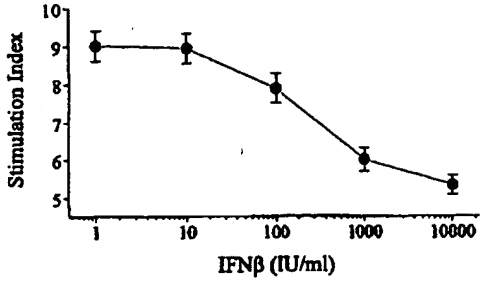

Fig. 4. Inhibition of in vitro p139–151 autoreactivity by IFNβ. Proliferative responses of LNC from (SWR×SJL)F_1 mice immunized 8 days prior with PLP 139–151. Various doses of IFNβ were added with 20 μM p139–151 at initiation of 3-day cultures. Error bars show ±S.D.

controls. In addition, fewer IFNβ-treated mice showed multiple relapses, 5/13 (39%) and 3/12 (25%) mice in the IFNβ-5000 and IFNβ-10000 treatment groups, respectively, compared to 11/12 (92%) control mice. Statistical analysis of mice surviving 14 days after initial EAE onset showed no significant difference in ER between IFNβ-5000 and IFNβ-10000 treatment groups. In addition to the therapeutic effect on ER, treatment of (SWR × SJL)F_1 EAE mice with IFNβ significantly prolonged time to first relapse ($p = 0.001$, logrank test). Mice treated with IFNβ-5000 and IFNβ-10000 relapsed more slowly with a median time to first relapse of 29.0 days vs 21.0 days for PBS-treated control mice.

3.5. IFNβ treatment of EAE produces a long-term improvement in mean clinical score and a delay in progression to disability

A marked improvement in mean clinical score was observed by day 56 in mice treated at onset of EAE with 5000 or 10000 IU IFNβ every other day (Fig. 3). At day 56, the mean clinical score was 3.7 ± 0.31 S.E. in PBS-treated mice compared to 3.0 ± 0.35 S.E. and 2.3 ± 0.34 S.E. in the IFNβ-5000 and IFNβ-10000 treatment groups, respectively.

3.6. IFNβ inhibits p139–151 T cell responses in vitro

We addressed the question of whether IFNβ inhibits autoreactive T cell responses. Eight days after immunization with p139–151, LNC from p139–151 immunized (SWR × SJL)F_1 mice were stimulated in vitro with p139–151 in the presence of various doses of IFNβ. When added with peptide at the initiation of culture, IFNβ inhibited the proliferation of determinant-primed LNC in a dose-dependent manner (Fig. 4). Inhibition did not appear to be due to IFNβ-mediated cytotoxicity since viable cell yields from each dosage group were similar (data not shown).

3.7. IFNβ-treated EAE mice show inhibition of p139–151 DTH but not proliferation

To determine whether treatment with IFNβ inhibited autoreactivity in vivo, p139–151-specific DTH ear swelling was measured in control and IFNβ-10000-treated mice 28

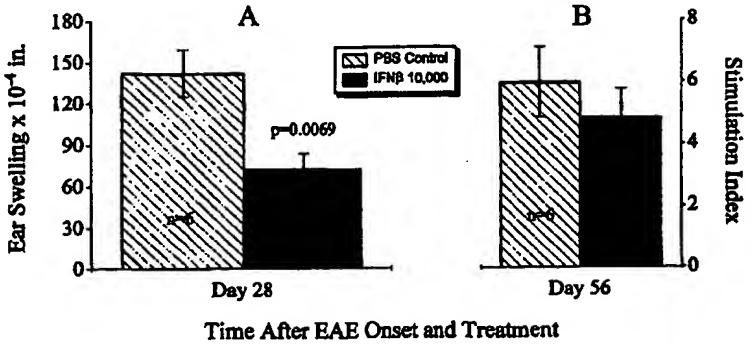

Fig. 5. Immunoreactivity to the priming p139–151 determinant in (SWR × SJL)F_1 EAE mice treated with IFNβ or PBS. (A) DTH ear swelling to p139–151 28 days after EAE onset and treatment. Mice were injected s.c. in one ear with 10 μg p139–151 and in the contralateral ear with 10 μg BSA. Ag-specific DTH was measured 24 h later as described in Materials and methods. A significant difference ($P = 0.0069$) in p139–151 DTH occurred between IFNβ-treated and control mice at 28 days after EAE onset and treatment. Error bars show ±S.E. (B) A significant difference in p139–151 proliferation by spleen cells was not apparent between both groups of mice 56 days after EAE onset and treatment. Error bars show ±S.E.

effect in EAE by altering the migration patterns of inflammatory T cells as has been previously suggested (Gresser et al., 1981).

Alternatively, the therapeutic effect of IFNβ may be due to immunomodulation. The immunomodulatory properties of IFNβ are not as obvious and as well studied as those of IFNγ but are readily apparent. Types I (α and β) and II (γ) interferons induce enhanced cell surface expression of class I MHC molecules, increased synthesis of immunoglobulins, enhanced pyrogenic activity, and activation of macrophages, NK cells, and cytotoxic T cells (reviewed by Borden, 1992; Sen and Lengyel, 1992; Farrar and Schreiber, 1993). In some cases the immunomodulatory effects of IFNβ diverge from those induced by IFNγ. Whereas IFNγ has been shown to enhance the expression of class II MHC on all cells tested, IFNβ has been shown to reduce class II MHC expression on human astrocytes (Ransohoff et al., 1991), human endothelial cells (Miller et al., 1994), and on murine macrophages (Ling et al., 1985). Using a variety of human antigen-presenting cells, Jiang et al. (1994) have recently demonstrated that IFNβ-mediated decreased expression of class II MHC results in a marked reduction in antigen-specific T cell responses. Thus, differential immunomodulatory effects of types I and II interferons may provide the basis for explaining the therapeutic effects of IFNβ and the well-documented pathogenic effects of IFNγ (Panitch et al., 1987) in MS treatment.

A limited number of recent studies suggest that IFNβ may have anti-inflammatory properties capable of inhibiting autoreactivity. Noronha et al. (1993) showed that IFNβ-induced inhibition of T cell proliferation was accompanied by down-regulation of IFNγ production suggesting a regulatory role of type I interferons on the expression and production of type II interferons. Peppler et al. (1994) showed that IFNβ-mediated inhibition of mitogen-induced proliferation of human peripheral blood lymphocytes in vitro was accompanied by enhanced expression and secretion of IL-10, an anti-inflammatory cytokine that inhibits the production of IFNγ from encephalitogenic T cells (Van der Veen and Stohlman, 1993). These data suggest that IFNβ may act in such a way as to modify the inflammatory cytokine profile of autoreactive T cells.

One or more of the diverse physiological properties of IFNβ may contribute to the inhibition of pathogenic autoreactivity directed against the priming p139–151 determinant. In addition, efficacy may be achieved by inhibiting the acquisition of new self-determinant recognition. Determinant spreading is a pathogenic process that accompanies and perhaps is responsible for chronic progression of autoimmune disease (Lehmann et al., 1992; Lehmann et al., 1993). It is possible that IFNβ alters autoimmune disease processes by preventing the development of the 'acquired' T cell repertoire in addition to its impact on the pathogenicity of the established p139–151 'memory' T cell repertoire.

It is of interest to note that there was a clear histopathological improvement in the IFNβ-treated mice but that the improvement was far from a cure. A similar significant but modest long-term therapeutic effect on severity and incidence of CNS inflammation has been described in RRMS patients treated with IFNβ (Interferon-β Multiple Sclerosis Study Group, 1994). Thus, the therapeutic impact of IFNβ-treatment clearly leaves room for improvement in the development of new protocols for treating autoimmune demyelinating disease and suggests that combined complementary therapies may be built around IFNβ.

The enhanced expression of relapse in (SWR × SJL)F_1 mice makes it a useful animal model for assessing the effectiveness of treatment protocols for inhibiting autoimmune demyelinating disease. The basis for the increased exacerbation rate in (SWR × SJL)F_1 mice is unclear but may be the consequence of combining complementary susceptibility genes from each parental strain. Microsatellite linkage analysis has suggested the presence of many genes in EAE susceptibility (Rossenwasser et al., 1994). Thus, it does not seem surprising that the F_1 offspring of appropriate EAE susceptible mouse strains may acquire complementary genes from both parents resulting in an increased susceptibility to autoimmune relapse. In addition, enhanced determinant spreading as a result of dual IA^s/IA^s-restricted antigen-presentation may also contribute to the increased exacerbation rate observed in (SWR × SJL)F_1 mice (Sobel et al., 1991; Tuohy and Thomas, 1995).

The present study indicates that the (SWR × SJL)F_1 mouse EAE model is appealing for studying MS because of its enhanced exacerbation rate, the consistent progression from relapsing disease to chronic disability, the development of chronic CNS demyelination, and because IFNβ is effective in treating both MS and EAE in (SWR × SJL)F_1 mice. Thus, we now have a unique opportunity for determining the therapeutic mechanisms responsible for the beneficial effects of IFNβ in autoimmune demyelinating disease. Such information will form a rational basis for combining complementary protocols with potential for having additive or synergistic therapeutic effects in the treatment of autoimmune-mediated demyelination widely believed to be occurring in MS.

Acknowledgements

This work was supported by National Multiple Sclerosis Society grant JF-2045 (V.K.T.), by National Institutes of Health grants NS-29095 (V.K.T.) and NS-29818 (B.D.T.), and by Cleveland Foundation grant #93-0641 (V.K.T.).

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Immunology and Cell Biology (1998) **76**, 483–496

Review Article

Early pregnancy factor: An extracellular chaperonin 10 homologue

HALLE MORTON

University of Queensland, Department of Surgery, Clinical Sciences Building, Royal Brisbane Hospital, Brisbane, Queensland, Australia

Summary Early pregnancy factor (EPF) has been identified as a homologue of chaperonin 10 (cpn10) with immunosuppressive and growth factor properties. As a homologue of cpn10, it belongs to the heat shock family of proteins (hsp) but, unlike other members of this family, EPF is detected extracellularly. Early pregnancy factor was first discovered in pregnancy serum by the rosette inhibition test, and the novelty of its discovery was that its presence could diagnose pregnancy within 6–24 h of a fertile mating. As well as being a monitor of the presence of a viable embryo, it is necessary for embryonic survival. In this capacity it acts as both an immunosuppressant and growth factor. Early pregnancy factor is also a product of proliferating primary and neoplastic cells and functions as an autocrine growth factor both *in vivo* and *in vitro*. It has a modifying effect on the outcome of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. Early pregnancy factor is considered to be one of the major factors involved in the modification of multiple sclerosis observed during pregnancy.

Key words: chaperonin 10, early pregnancy factor, experimental autoimmune encephalomyelitis, growth factor, heat shock proteins, immunoregulation, lymphokines, pregnancy, pregnancy proteins, rosette inhibition test.

Introduction

Pregnancy has provided a number of challenging problems for immunology, the first of which comes with the recognition that the foetus is antigenically alien to the mother.[1] Conversely the relationship of the mother to the foetus is potentially the same as that in which a graft-versus-host reaction might be expected.[2] To resolve this dilemma, it was originally argued that the foetus occupied an immunologically privileged site.[3,4] Privileged sites, such as the eye, are certainly known, and privilege presumably refers to anatomical locations free from humoral and cellular attack. On the face of it, both pre- and post-implantation states would be unlikely to qualify for this form of immunological exemption.

It was this immune exemption of the developing embryo that captured our attention as a model in the search for a more appropriate form of immunosuppression than was available some 25 years ago. It seemed logical that there may be a subtle suppression of the maternal immune response by serum factors that would not endanger the life of the mother but would prevent an immune response against the foetus.

During the 1960s, anti-lymphocyte serum (ALS) was examined as a tool to prevent rejection of transplanted kidneys.[5] The genesis of the rosette inhibition test, developed by Bach *et al.*,[6] resulted from the need for a suitable test to assess the potency of ALS used for immunosuppression. We reasoned that if there was an immunosuppressive factor present in pregnancy serum that prevented the mother from rejecting the antigenically alien foetus, it should augment the rosette inhibition titre of an immunosuppressive ALS. This was found to be the case and thus early pregnancy factor (EPF) was first described.[7,8]

What was entirely unexpected was that EPF appeared in serum within 6–24 h of fertile mating.[8] Before the discovery of EPF, it was considered that the embryo was a silent passenger during the pre-implantation period, and maternal recognition of pregnancy did not occur until implantation.[9] Indeed, the earliest indication prior to this of an altered physiology in pregnancy was the production of human chorionic gonadotrophin (hCG).

With the discovery of EPF it became apparent that the maternal system was preparing for implantation and successful pregnancy from the moment of fertilization. Studies with specific antibodies have shown that EPF is essential for the growth and survival of the embryo both during the pre- and post-implantation periods.[10,11] Early pregnancy factor has been shown to be an immunosuppressant[12,13] and growth factor,[14,15] and it is by these means that it acts to ensure the well-being of the foetus. The notion that EPF has a role in embryonic development other than immunosuppression was supported by the finding that it is present in fertile hen's eggs as well as in the developing chick (H Morton, F Ellendorff, DJ Morton unpubl. data, 1981).

A role for EPF is not confined to pregnancy. It is a product of proliferating primary[16] and tumour cells[14,15]

Correspondence: Dr Halle Morton, University of Queensland, Department of Surgery, Clinical Sciences Building, Royal Brisbane Hospital, Brisbane, Qld 4029, Australia.

Received 31 July 1998; accepted 31 July 1998.

where it acts as an autocrine growth factor. Early pregnancy factor is also a product of platelet activation[17] and it is proposed therefore that it may play a part in wound healing.[18] The properties of EPF that have been determined to date suggest that it may have a role as a cytokine.

In the early 1990s EPF was purified from human platelets and found to be highly homologous with chaperonin 10 (cpn10), a member of the heat shock family of proteins (hsp).[19] This was most surprising because cpn10 is essentially an intracellular protein which acts together with chaperonin 60 (cpn60) within the mitochondria to mediate protein folding.[20]

The present review will discuss EPF as an extracellular homologue of cpn10, explore its activity in the rosette inhibition test and discuss its immunological and growth factor properties determined to date.

Early pregnancy factor identified as a homologue of chaperonin 10

Since we identified EPF, purified from human platelets, as cpn10, we have prepared recombinant[18] (and H Morton *et al.*, unpubl. data 1998) and synthetic[21] protein and shown that these molecules are fully active in the rosette inhibition test, the bioassay for EPF.[8] Native cpn 10, purified from rat liver mitochondria (gift of Dr Peter Hoj, University of Adelaide, SA, Australia) is also active in the assay. In contrast, *Escherichia coli* cpn10 (known as GroES)[22] which has ~40% similarity with rat cpn10, exhibited no activity in the rosette inhibition test. This result was consistent with our observation that *E. coli*-conditioned medium is not active in the assay while medium conditioned by all mammalian cell lines tested,[14] as well as by yeast cells (MJ Hoskin *et al.* unpubl. data, 1991), is active.

The activity of these different forms of EPF/cpn10 in the rosette inhibition test is neutralized by antibodies used in previous studies.[10,14,16] Furthermore, polyclonal affinity-purified IgG antibodies have been prepared against synthetic peptides corresponding to residues Ac1–11 (anti-N) and 34–44 (anti-I) of rat and human EPF/cpn10.[23,24] Activity detected by the rosette inhibition test in pregnancy serum,[8] rat serum following partial hepatectomy,[16] and medium conditioned by actively dividing tumour cells[14] is neutralized by these antibodies.

Early pregnancy factor/cpn10, like other hsp, appears to be a member of a large gene family, distributed across many chromosomes.[25] Several transcripts have been detected, indicating that more than one of these genes is likely to be functional. In addition, the cDNA transcribed from mRNA, encoding cpn10 from rat liver[26] and human melanoma cells (RM Murphy *et al.* unpubl. data, 1995), does not code for a classic secretory signal sequence. Early pregnancy factor joins a growing list of proteins that are secreted from the cytoplasm by unknown mechanisms rather than by the usual route through the endoplasmic reticulum.[27]

Heat shock proteins in the immune response

Chaperonin 10, as a chaperonin, belongs to the broader group of hsp, otherwise known as stress proteins.[28] Heat shock protein responses have been observed in every cell type examined and they are among the most conserved proteins known in phylogeny with respect to both structure and function.[29] It is not surprising, therefore, that they are also involved in the immune response.[30–33] They have a role in assembly of immunoglobulins,[34] antigen processing[35,36] and presentation of antigen to T cell receptors,[37] lymphocyte homing[38,39] and autoimmune disease.[40] In these roles, hsp act intracellularly or are expressed on the cell surface.

Stress can induce the movement of these proteins to different cellular compartments[40,41] and, in some cases, outside the cell. Hightower and Guidon described the release of several hsp (hsp110, hsp71, hscp73) from cultured rat embryo cells.[42] These authors concluded that the rapid release of stress proteins is a homeostatic mechanism for the transfer of some of these proteins from cells capable of mounting a strong stress response to neighbouring cells that cannot. Early pregnancy factor is another example of a hsp, released from the cell, that acts extracellularly in an autocrine, paracrine and/or endocrine fashion.

Bacterial hsp 60 and hsp70 are highly immunogenic, and immune responses to these antigens have been noted in a number of infectious diseases.[37] Prokaryotic hsp60 and hsp70 share significant sequence homology with related stress proteins in mammalian cells. This finding led to the suggestion that these antigens may also function as targets of the immune response, and their potential for inducing autoimmune disease has been widely explored. Induction of autoimmunity by hsp was not reported in most experimental systems, rather they appeared to have a modifying effect on the course of autoimmune disease.[42–45]

Several groups have shown that hsp10 (cpn10) may also have a role as a modulator of autoimmune diseases. Ragno *et al.* studied the effect of a synthetic hsp10 from *Mycobacterium tuberculosis* on the early response to induction of adjuvant-induced arthritis in Lewis rats.[46] The mycobacterial hsp10 promoted a suppressive response. In this model, modulation of adjuvant arthritis was specific for the mycobacterial form of the molecule because neither GroES (the *E. coli* homologue) nor rat hsp10 (cpn10) elicited any protection. Furthermore Ben-Nun *et al.* described a protective role for a 12kDa protein of *M. tuberculosis* in a model of experimental autoimmune encephalomyelitis (EAE),[47] and Ferrero *et al.* showed that a protein belonging to the GroES class is a protective antigen in mucosal infection in mice.[48] Thus in various models of experimental autoimmune disease, it was demonstrated that hsp could offer protection against subsequent induction of disease.[44] Van Eden *et al.* concluded that hsp have a uniquely protective quality, probably due to their stress-inducible nature, and it is the recognition of self-hsp molecules that contributes to maintenance of self-tolerance.[44]

Nature of the early pregnancy factor molecule and factors affecting its activity

There has been much discussion in the literature and elsewhere over the years about the true nature of the EPF molecule. We have now unequivocally established

the identity of EPF as a homologue of cpn10, with a molecular mass of 10843.5 Da.[19]

Several authors have reported purification of EPF of different molecular size,[49–53] identified by activity in the rosette inhibition test (see following). The dose–response curve of EPF in this assay is bell shaped with high as well as low concentrations giving negative results; as the concentration of EPF decreases, the curve moves to the left. This characteristic of EPF in the assay can lead to the most active fractions being overlooked during fractionation of complex biological mixtures, with the fractions selected containing protein with very low activity or predominantly inactive protein with small amounts of EPF.[54] None of these purified products discussed here have been available in sufficient amounts to determine their amino acid sequence.

Previous studies have shown that the oviduct, during oestrus and early pregnancy, produces an inhibitor,[55,56] which modifies the response of EPF in the rosette inhibition test.[54] In its presence, higher concentrations of EPF are required to stimulate release of lymphokines by which the action of EPF in the rosette inhibition test and the delayed-type hypersensitivity (DTH) reaction is mediated.[57,58] The range of concentrations over which EPF is able to affect lymphocytes is thus extended, suggesting a possible biological function for the inhibitor. A larger molecular binding protein(s) also associates with EPF in serum during the pre- and peri-implantation period. It appears to act as a storage protein for EPF and has been observed in mice,[59] sheep,[60] pigs[61] and humans.[62] Early pregnancy factor can be separated from the latter by anion exchange chromatography[60] but activity is not altered during this process. These results are in contrast with the properties of the inhibitory substance discussed here but, when taken together, suggest a mechanism of both course (storage/protein carrier) and fine (inhibitory protein carrier) regulation of EPF activity. An analogous situation has been described for control of insulin-like growth factors (IGF). Large and smaller binding proteins have been identified, with the former binding to the majority of IGF in serum and performing a storage function, while the latter is itself subject to hormonal control, which in turn closely regulates IGF function.[63]

Clarke has suggested that activity attributed to EPF in the rosette inhibition test is a result of interaction of thioredoxin with oestrous serum.[64] They showed that, while isolated thioredoxin on its own has no EPF activity, activity was observed in combination with oestrous serum.[64] They concluded that thioredoxin was not EPF but was involved in the EPF system. Lash, on the other hand, demonstrated that the ability of thioredoxin to modulate EPF activity mimicked the action of the oestrous oviduct-derived inhibitor.[65] However, she concluded that thioredoxin must be operating through molecules other than EPF because the EPF/cpn10 molecule does not contain any cysteine residues essential for the redox activity of this molecule.[65] We have shown that in the assay EPF initiates a cascade reaction, and it is possible that thioredoxin could well be involved in downstream events. Now that pure EPF protein is available, these possibilities can be tested.[66]

The rosette inhibition test, the bioassay for early pregnancy factor

Central to the research has been the use of the rosette inhibition test as the bioassay for EPF. This deserves some extended discussion because it has been a major stumbling block to rapid progress. Here we attempt to summarize the reasons why we have yet to displace this rather unwieldy biological assay with a more universally acceptable assay system, such as an ELISA.

As mentioned earlier, the rosette inhibition test was the assay by which EPF was first discovered. Despite its limitations and despite being very technically demanding, it still remains the only reliable assay for detecting EPF at biological concentrations. From our experience, the assay detects very low picomolar concentrations of EPF. Thus it is exquisitely sensitive and in general below the levels of detection by conventional assay methods. The presence of EPF as a diagnosis of pregnancy in any species can be determined either directly by testing lymphocytes from the subject or indirectly by testing allogeneic or heterogeneic lymphocytes, following incubation in the test sample.[67] To test lymphocytes directly, a species-specific ALS must be available. While this is practicable in mice, humans, pigs, and cattle, in some of the more exotic species (elephants, pandas, kangaroos), serum samples must be tested in the mouse assay system. While the rosette inhibition test is a most reliable test for diagnosis of pregnancy, particularly in the very early pre-implantation stage, it is too laborious for use as a routine pregnancy test.

In spite of the cumbersome nature of the assay, many groups have used it in a research capacity for specific investigations. Results have shown that EPF is present in serum of all species tested including mice,[8] humans,[67–74] sheep,[75] pigs,[61,76] cattle[77,78] and red deer.[79] A diagrammatic representation of the assay is shown in Cavanagh and Morton.[80]

The assay has been criticized, the main criticisms being that it is difficult to perform, it is non-quantitative and it is non-specific. The assay is very sensitive to water quality and is readily negated by even trace amounts of agents that affect the lymphocyte cell surface. Once the assay is meticulously set up, using glass distilled water and disposable plastic ware, results obtained are very reliable and repeatable.[81] The assay is non-quantitative but can be used in a semiquantitative manner by comparing the highest dilution of a sample to give a positive response in the assay. However, as mentioned earlier, samples cannot be compared in this way in the presence of the inhibitor.

The specificity of the rosette inhibition test for EPF has been questioned. In the past, various other pregnancy-specific proteins were said to be positive in the rosette inhibition test, for example hCG and human placental lactogen (hPL), but this activity was later shown to be due to contamination of the substances with EPF.[62] More recently Lash and Legge have investigated known proteins with growth factor activity using the rosette inhibition test over a wide range of dilutions.[82] The proteins tested were insulin, insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II), prolactin, hPL and growth hormone. Of the six proteins tested, IGF-I demonstrated

activity in the assay, over the same dilution range associated with EPF. These authors found that serum samples and conditioned medium with EPF activity did not have detectable levels of IGF-I in a radio-immunoassay (RIA), although this may have reflected the limitations of the sensitivity of the RIA; IGF-I in the rosette inhibition test was active at picomolar concentrations whereas the RIA only detected down to nanomolar concentrations. They questioned whether in fact EPF was IGF-I. Cavanagh and Morton have provided evidence that disproves this suggestion.[19] Experiments demonstrated that antibodies, prepared against an N-terminal peptide (Ac1–11) and a peptide synthesized to correspond to an internal amino acid sequence (34–44) of the EPF/cpn10 molecule[18,24] could neutralize the activity detected by the rosette inhibition test in 24-h mouse pregnancy serum, 6-day gestation human pregnancy serum, rat serum 24 h post-hepatectomy and medium conditioned by the Madin Darby bovine kidney (MDBK) cell line (ATCC CRL22).[18] Furthermore, in the presence but not in the absence of ATP, immobilized cpn60 could remove all activity in the rosette inhibition test from human pregnancy serum (6-day gestation) and activity could be recovered by removing ATP from the immobilized complex; this assay demonstrates functional activity of cpn10.[20] The results show that the molecule, detected in pregnancy serum by the rosette inhibition test, is cpn10.

While we do not know at this stage the exact mechanism involved in the rosette inhibition test, we do know that the result of EPF binding to T cells initiates a cascade reaction (Fig. 1). At least two lymphokines have been detected, both of which are active in the assay. These are discussed in more detail in the following section.

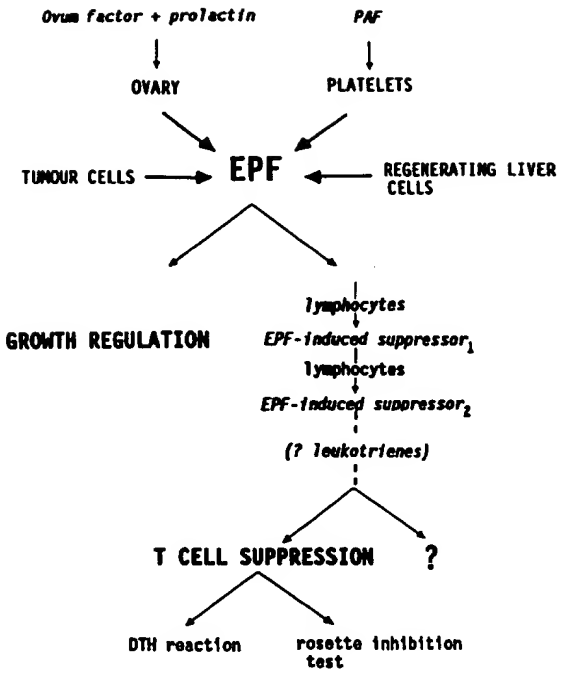


Figure 1 Flow chart demonstrating sources of early pregnancy factor (EPF), target cells and proposed roles. Production of EPF is induced from ovaries by ovum factor[132] (a product of the fertilized ovum) plus prolactin, from platelets by platelet-activating factor (PAF) and has an endocrine role. Early pregnancy factor is secreted by proliferating tumour cells and regenerating liver cells (following partial hepatectomy) and has an autocrine/paracrine role. (Reproduced with permission from Morton *et al.*[133]) DTH, delayed-type hypersensitivity.

Time course of early pregnancy factor in serum following i.p. injection

Because the rosette inhibition test is the only assay that can detect EPF in serum, it has been used to determine the time course of activity *in vivo* following injection of 1 μg i.p. into mice.[18] Platelet EPF[19] and synthetic EPF,[21] synthesized to correspond with the amino acid sequence of human EPF/cpn10 and N terminally acetylated, gave a half-life of activity in serum of 6.4 and 5.8 days, respectively. The prolonged half-life was not due to acetylation of the molecule because the non-acetylated synthetic molecule gave a value of 5.6 days. In contrast, following injection of recombinant EPF, prepared using the plasmid pGEX-2T expression system[18] (and H Morton *et al.*, unpubl. data 1998), activity had disappeared from serum within 6 h. The amino acid sequence of pGEX-2T rEPF is identical to human cpn10 with an additional G-S-M at the N terminus and it is not acetylated. We postulate that the long half-life of the native and synthetic EPF in serum is due to their ability to bind to serum protein(s) through the N terminus, and the altered N-terminal sequence of the rEPF abrogates this binding. To investigate this further, we tested the half-life of two peptides derived from the EPF amino acid sequence, an N-terminal peptide (Ac1–11; N-peptide) and an internal peptide (34–44; I-peptide). Both peptides are active in the rosette inhibition test.[18] The half-life of activity following i.p. administration of N peptide to mice was found to be 7.4 days while that of the I-peptide was 4.3 h. These results supported our proposal that EPF binds to serum proteins through the N terminus, thus extending the time course of its activity in serum. However, following therapeutic abortion, EPF activity disappears very rapidly from serum, with negative values being reached within 6–48 h after surgery.[83,84] Loss of EPF activity in serum was more rapid than that of other pregnancy-associated substances.[84] The mechanism by which the half-life of EPF activity in serum is reduced from ~6 days to 5 h following abortion is yet to be determined. Because EPF modifies the mother's immune system, it certainly would be to her advantage to abrogate this activity as soon as possible after abortion, at a stage during which she would be most vulnerable to infection.

Early pregnancy factor induces genetically restricted lymphokines

The immunosuppressive action of EPF is mediated through the sequential induction of lymphokines. Rolfe *et al.* have described two lymphokines, EPF-S_1 and EPF-S_2, induced from both human and mouse lymphocytes.[57,58,85] The activity of both factors is genetically restricted, a property that clearly distinguishes them from the inducing EPF, which is neither strain nor species restricted. These factors

were designated suppressor factors on the basis of their ability to suppress the DTH reaction, a well-established measure of T cell activity. Evidence has been presented that indicates that the release of these factor(s) is induced by EPF, acting by classical endocrine pathways which use cAMP as a second messenger.[57,86] Furthermore like other antigen non-specific suppressor factors,[87] their release can be stimulated by Con A.

Different cell populations are involved in production of EPF-S_1 and EPF-S_2, with EPF-S_1 having the ability to induce EPF-S_2 but not vice versa.[85] Restriction of activity of EPF-S_1 has been mapped to the I region of the murine H-2[57] and to HLA-DR in humans,[58] while that of EPF-S_2 is localized to the Igh region.[85] In mice, spleen cells producing EPF-S_1 and those responding to it shared identity at either I-A, I-B or I-J subregions,[57] and EPF-S_2 (but not EPF-S_1) carries the I-J determinant.[85] The significance of the genetic restriction of these factors during pregnancy is yet to be determined but it would appear that any downstream immunological responses, initiated by EPF binding to lymphocytes, is directed towards cells of the same genetic lineage.

EPF-S_1 and EPF-S_2 are detected by the rosette inhibition test. Lymphocytes are incubated with EPF, then the inducing EPF is removed from the conditioned medium by immobilized anti-EPF IgG and the absorbed supernatant is tested for EPF-S_1 and EPF-S_2 with cells of appropriate genetic background. Absorption of EPF is monitored by testing with cells that do not share identity at the I region or Igh. While both EPF-S_1 and EPF-S_2 can be detected *in vitro*, only EPF-S_1 can be detected in mouse pregnancy serum. Several explanations are possible. Either EPF-S_2 has a very short half-life in serum, or reactions involving EPF-S_2 may proceed in the lymphoid tissue by cell–cell contact.

Early pregnancy factor as an essential survival factor for the embryo

While EPF is a marker for the presence of a viable embryo, it is also necessary for its continued survival. Studies by Athanasas-Platsis *et al.* and Igarashi have shown that passive immunization of pregnant mice with monoclonal or affinity purified polyclonal antibodies to EPF will terminate pregnancy.[10,11,88] Athanasas-Platsis *et al.* went on to show that treatment of mated mice with antibodies to EPF as early as 8 h post coitum (p.c.) affected development of the majority of embryos at around the 1–2-cell stage, leading to retardation in embryonic development with subsequent failure to implant.[11] Treatment on days 2 and 3 p.c. or days 3 and 4 p.c. did not affect embryonic development but did inhibit implantation. These results suggested that there are two temporal windows during early pregnancy where EPF is, in part, instrumental in initiating or participating in events crucial for the development and implantation of the early embryo. In contrast, EPF-S_1 appears to be crucial to embryonic survival only at implantation. Passive transfer of pregnant mice with a monoclonal antibody to EPF-S_1 was only effective in disrupting pregnancy if administered at the time of implantation.[89] The significance of this finding is under investigation.

The role of EPF, apparent during the early stages of cleavage, is indirect rather than direct, as morphological development proceeded uninterrupted to the blastocyst stage when two-cell stage embryos were cultured *in vitro* in the presence of anti-EPF antibodies.[11] Moreover, measurements of rate of protein synthesis, a very sensitive assay of physiological state, were not affected by the presence of anti-EPF IgG. Early pregnancy factor may be acting indirectly at this stage, either by initiating systems *in vivo* which are important to embryonic survival or by suppressing systems, for example lymphokine/cytokine production, which would damage the developing embryo.[90] Early pregnancy factor may be important in regulation of cytokine production either directly or through induction of specific lymphokines.

While development of early embryos progressed uninterrupted to the blastocyst stage *in vitro* in the presence of anti-EPF antibodies, the development to the outgrowth stage was inhibited when morulae were cultured for 168 h in the presence of monoclonal anti-EPF IgM (Fig. 2). It is at the morula/blastocyst stage that the embryo develops the capacity to produce EPF.[81] The inhibition of trophoblast outgrowth or, in a high proportion of cases, death of the blastocyst, coincides with embryonic production of EPF, suggesting that it is a necessary autocrine growth factor at this stage of development.

Further evidence of a growth factor role for EPF in embryonic development has been demonstrated. Studies by Drs Hill and Brenneman at the National Institute of Child Health and Human Development (NICHD), National Institutes of Health, USA (NIH), have established that EPF appears to be an endogenous growth regulator of the early post-implantation embryo (S Lee *et al.* unpubl. data, 1998). Treatment of whole, cultured E9.5 mouse embryos with exogenous EPF stimulates growth. This effect is overcome by simultaneous addition of anti-EPF antibody (anti-N-peptide antibody; see earlier), while anti-EPF alone has a growth inhibitory effect.

The communication network that exists between the endocrine and the immune systems during pregnancy is well illustrated in the action of EPF. The mechanism of action is purely speculative at this stage but the paracrine action of EPF during early embryonic development may be necessary for immunoregulation of maternal anti-foetal reactions, while the latter autocrine action may be necessary for development and invasion of the trophoblast and growth of the implanted embryo.[13] However, we postulate that EPF still maintains its immunosuppressive role in addition to its growth factor role at implantation and beyond.

EPF as a survival factor/growth factor for proliferating primary and tumour cells

Early pregnancy factor was first shown to be associated with tumour cell growth by Rolfe *et al.* who demonstrated, with the rosette inhibition test, that EPF could be found in the serum of patients with germ cell tumours of the testis but not in patients with non-germ cell tumours, benign testicular disease or in the serum of healthy male controls.[91] Mehta and Shahani also found that the presence of EPF in

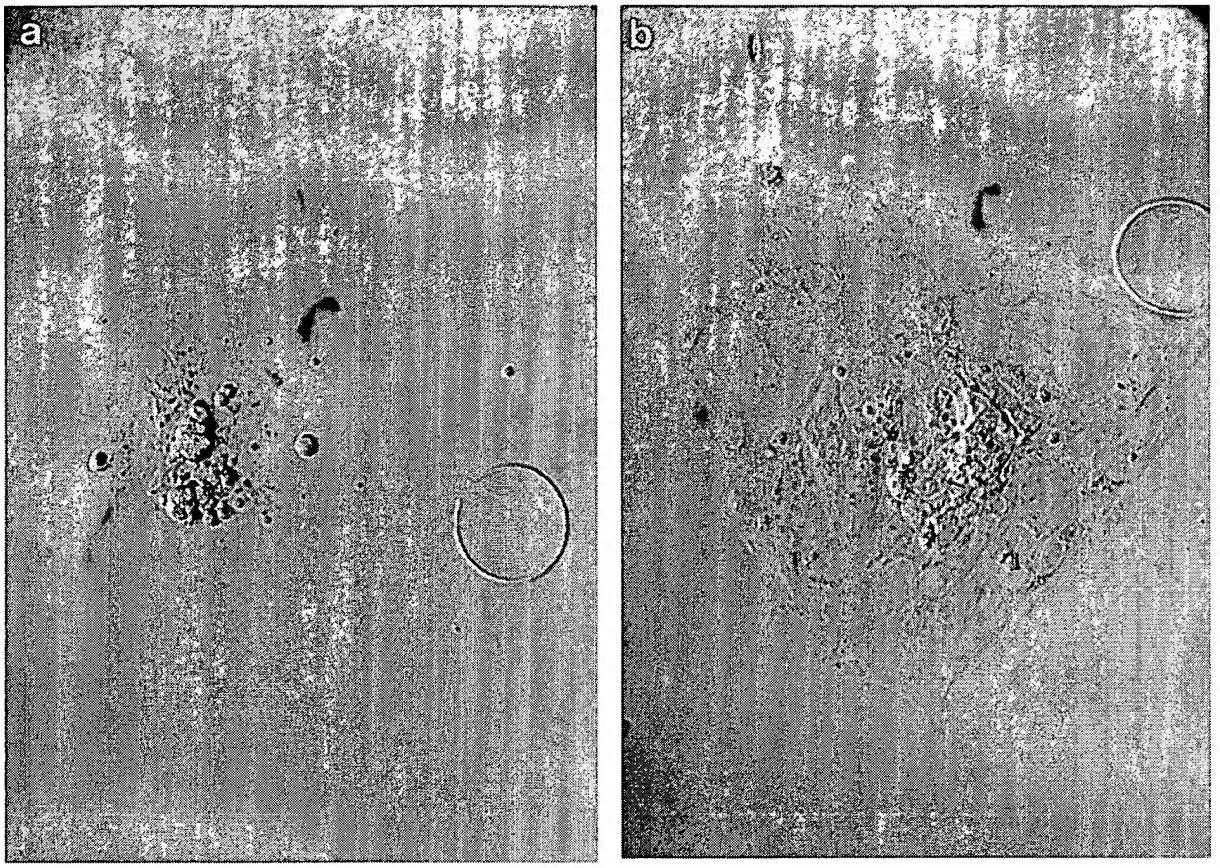


Figure 2 The appearance of outgrowths of embryos after culture for 168 h in the presence of (a) 2.5 μg/mL anti-early pregnancy factor (EPF) monoclonal IgM 5/341 or (b) 2.5 μg/mL isotype control antibody. Morulae were collected from mice 56 h post coitum (p.c.), washed extensively in BMOC-2/BSA/10% foetal bovine serum (FBS), supplemented with 20 mmol/L glutamine and 375 μg/mL sodium bicarbonate (BMOC/FBS) and then cultured one per 5 μL droplets of BMOC/FBS containing either 2.5 μg/mL monoclonal anti-EPF IgM 5/341 or 2.5 μg/mL isotype control antibody (monoclonal IgM 7/331).[10] After 168 h in culture, development of the outgrowth stage was recorded. The area of outgrowth with the isotype control antibody did not differ from that with culture medium alone. (Reproduced with permission from Athanasas-Platsis.[13])

serum could distinguish between patients with choriocarcinoma, whose serum tested positive for EPF, and those with hydatidiform mole, where no serum EPF activity was detected.[92] Due to the time-consuming assay, these studies have not been followed up on a large scale so it has not been confirmed whether the results were qualitative or quantitative. This will have to await the development of a very sensitive immunoassay for detection of EPF in serum. However, subsequent work has been shown that EPF is a product of actively dividing tumour and transformed cells both *in vitro* and, in the mouse, *in vivo*.[14]

As well as a role as an autocrine growth factor in the survival of embryonic cells, EPF plays a similar role in tumour cell growth. Not only do proliferating tumour cells produce EPF, they also have an active requirement for its continued presence. Production ceases if cells undergo differentiation or growth arrest.[14] Experiments by Quinn *et al.* demonstrated that coculture of tumour cells with increasing doses of anti-EPF monoclonal antibodies resulted in a significant, dose-dependent decrease in rate of cell growth and viability.[14]

The effectiveness of the anti-EPF monoclonal antibodies in limiting the growth of tumour cells in culture could explain the problems encountered in producing high-affinity IgG monoclonal antibodies. Monoclonal antibodies produced by Quinn *et al.* were all low-affinity IgM antibodies.[14] Hybridomas were produced which secreted high-affinity anti-EPF IgG but these were inherently unstable and none survived the first cloning. Instability of anti-EPF-producing B cell clones was also demonstrated *in vivo*. While production of neutralizing polyclonal rabbit anti-EPF IgG was possible in the short term, this production could not be maintained. Six to 8 weeks after the initial immunization, antibody production ceased.[24]

Tumour cells also require EPF to maintain viability *in vivo*. Quinn and Morton inoculated CBA mice s.c. with transplantable MCA-2 tumour cells and showed that anti-EPF administration, over a 4-day period after tumour inoculation, was effective in limiting the size of the tumours when measured on days 9 and 13.[15] In the second set of studies, anti-EPF monoclonal antibodies were administered to mice with established tumours. This was a much more

stringent test of the anti-proliferative capacity of the antibodies because at this stage the tumour burden was increased substantially and the tumour had established advantageous interactions with the host, such as extensive vascularization. Determination of the rate of DNA synthesis by the tumours in these experiments provided conclusive evidence that the anti-EPF monoclonal antibodies were inhibiting the growth of established tumours. That a decrease in the serum EPF titre was associated with inhibition of tumour DNA synthesis confirmed that EPF is required by tumour cells to maintain optimal levels of cell division.

The limitation of tumour cell growth *in vitro* is apparently due to EPF-specific antibodies interfering with a mechanism that acts directly to maintain the cells in the active phases of growth.[14] *In vivo* there may be additional host mechanisms that also act to limit the tumour growth after anti-EPF administration.[15] The capacity of EPF to initiate a cascade reaction, the soluble products of which are active in the EPF bioassay, has been established.[57] Furthermore the presence of EPF-induced lymphokines in the serum of CBA mice bearing MCA-2 tumours has been confirmed (BE Rolfe, pers. comm. 1989). Studies analysing the reaction of the host's immune system against syngeneic transplantable tumours have described the induction of suppressor cells.[93] The relevance of the EPF-induced lymphokines to survival of tumour cells *in vivo* has yet to be determined.

Early pregnancy factor is not only a product of proliferating tumour cells. Primary cells in culture will produce EPF when the cells are actively dividing. Quinn tested conditioned medium from primary cultures of rat pituitary cells, canine endothelial cells and rat fibroblasts.[94] After 5 days in culture, the cells were approaching confluence and EPF was present in the medium.

Quinn *et al.* demonstrated that production of EPF by primary cells is not just a phenomenon of cells in culture, released from the normal controls present *in vivo*.[16] Following partial hepatectomy (PH) in rats, EPF could be detected in serum within 8 h of surgery, peaking at 48 h, then disappearing by 3 weeks. After PH, the major wave of DNA synthesis starts within 14 h and reaches a peak by 24 h. The response is tightly regulated and is thought to involve interplay between growth stimulatory factors, operative in the prereplicative phase and inhibitory factors, which may be important in later stages as cell division ceases.[95] Liver mass is restored in rats with extraordinary rapidity, with the residual lobes (33% of the whole) nearly doubling in size by 48 h and approaching the original liver weight by 7 days after surgery. The time course of EPF in serum after PH demonstrated that production commences after the time (0–4 h) that hepatocytes, as a consequence of transition from G_o to G_1, become competent to divide. Early pregnancy factor is first detected during the time that the competent hepatocytes have been shown to be transversing from G_1 to S phase (starting 4 h after PH and lasting until 14–16 h), in preparation for the major wave of parenchymal cell DNA synthesis, which peaks at 24 h.[96]

Administration of EPF antibodies 18 h after PH resulted in a significant decrease in the uptake of [^{3}H]-thymidine by the liver remnant, measured from 22 to 24 h after PH. The early increase of EPF in serum after PH and the inhibitory effect of EPF antibodies on hepatic DNA synthesis indicate that EPF production is essential to the sequence of events that culminates in DNA synthesis and cell division.

While the induction of cpn10 or cpn60 in the livers of rats after PH has not been described elsewhere, there is evidence of expression of the heat shock protein 70 gene family, also classed as molecular chaperones, during liver regeneration.[28] Several groups have shown induction of mRNA for some members of this family in the 24 h after PH,[97,98] suggesting that heat shock proteins have not only the function of protection against various stresses but also physiological functions in normal cell growth and development.[97]

Cavanagh and Morton compared the biochemical and immunological properties of EPF, derived from the various sources, namely human pregnancy serum and urine, medium conditioned by ovaries from pregnant mice, medium conditioned by the bovine kidney cell line MDBK, regenerating rat liver, rat serum 24 h after PH and human platelets.[19] Biological activity of all positive sources followed the same pattern throughout the complex fractionation scheme, with no activity observed in control materials at any stage. As well as identical biochemical behaviour, the active agents from these sources exhibited similar immunological properties. In all instances, the final active fraction was bound by a monoclonal anti-EPF antibody and could be recovered quantitatively. While only platelets provided sufficient protein for detailed analysis, the identical immunological and biochemical behaviour suggests that the bioassay, the rosette inhibition test, is detecting a single substance or closely related family of substances, acting in diverse biological situations.[19] The common ground in these situations is the association with cell proliferation, of either primary, embryonic or tumour cells.

Immunology of early pregnancy factor

As mentioned earlier, EPF was first assigned an immunosuppressive role due to its ability to augment the rosette inhibiting properties of an immunosuppressive ALS. Further studies by Noonan *et al.* showed that it could suppress the DTH reaction, an *in vivo* measure of immune response.[12] This reaction is T cell dependent, as is the rosette inhibition test. These studies were repeated by Rolfe *et al.* using purified human EPF and once again, suppression of the DTH reaction was demonstrated.[62] This group went on to show that the suppression by EPF in this assay was not direct but was mediated by EPF-induced soluble factors (Fig. 3). As in the rosette inhibition test, the ability of these factors to suppress the DTH reaction was genetically restricted.

Athanasas-Platsis studied the effect of EPF on lymphocyte proliferation induced by the lectins, phytohaemagglutinin (PHA), Con A and pokeweed mitogen (PWM).[13] These lectins are polyclonal activators of T cells. Con A and PHA are selective T cell mitogens when compared to their effect on B cells, whereas PWM is both a T and B cell activator. Native EPF purified from human platelets[19] induced a significant inhibition of T lymphocyte proliferation induced by PHA (Fig. 4) and Con A, with the more marked effect observed with PHA.

In contrast, EPF augmented the IgM production of spleen cells activated with PWM (Fig. 5). A bell-shaped dose response was observed in these assays, with high as well as low doses of EPF resulting in a negative assay. A similar response was demonstrated in the rosette inhibition test.[50,54] This may reflect the fact that EPF, at higher concentrations, tends to form oligomers,[20] so possibly masking specific binding sites on the molecule. Early pregnancy factor may be acting directly on T cells, suppressing their response to specific T cell mitogens while enhancing the response to B cell mitogens. On the other hand, EPF may be acting indirectly by binding $CD4^+$ cells, initiating production of lymphokines, which in turn could suppress the autocrine/paracrine response of cells to T cell activators. In this case, the activated $CD4^+$ cells would enhance the B cell IgM production induced by PWM. The action of EPF on T cell populations is at present under investigation.

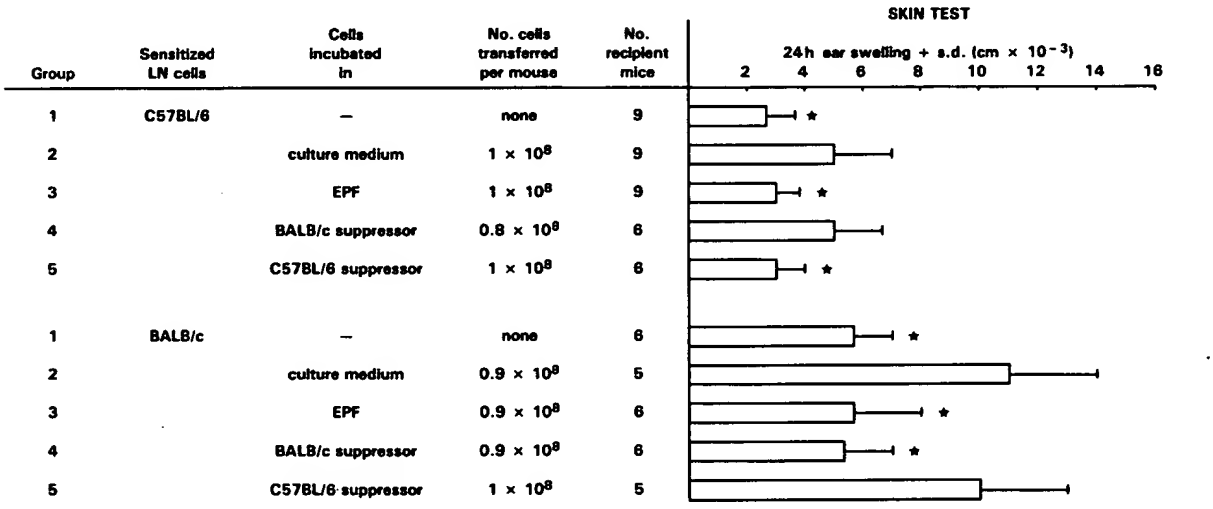
Antigen-induced proliferation of T cells is also suppressed by EPF. Lymph node cells, from rats previously inoculated with myelin basic protein (MBP) in adjuvants, were challenged with MBP in the presence or absence of synthetic EPF (H Morton *et al.*, unpubl. data 1998). The ability of these cells to proliferate in response to MBP was suppressed by EPF. The response was dose dependent. Further evidence of the ability of EPF to suppress MBP-activated lymphocytes was demonstrated by its ability to prevent the transfer of disease by lymph node cells from EAE rats to naïve syngeneic rats (H Morton, unpubl. data 1995). Cells were harvested from the draining lymph nodes of EAE rats, 10 days after inoculation with MBP in Freund's complete adjuvant, incubated with synthetic EPF (1 nmol) or vehicle alone, then transferred (10^8 cells/rat) to naïve, syngeneic rats. Early pregnancy factor prevented transfer of disease.

A further example of the suppressive effect of EPF on Th1 responses was demonstrated by its ability to prolong the survival time of allogeneic skin grafts in rats. Allogeneic skin grafts are known to induce activation of Th1 cells, producing IL-2 and IFN-γ. Prolongation of graft survival is associated with suppression of Th1 cell activation (diminished IL-2, IFN-γ) and activation of Th2 cells (increased IL-4, IL-10).[99] Studies by S Athanasas-Platsis (unpubl. data 1998) have shown that labelled EPF binds to a subpopulation of human $CD4^+$ T cells. This binding was specific because it was abrogated by cold EPF. Partial characterization of cloned $CD4^+$ EPF-binding cells showed that they were of the T helper/inducer phenotype.

Wegman *et al.* has proposed that in mice, Th1 responses are systemically suppressed during pregnancy, this suppression being accompanied by local expression of Th2 cytokines in placental tissue that may be beneficial for foetal survival.[100] Other workers have produced evidence that Wegmann's theory is also valid for humans.[101] We suggest that EPF has an important role in maintaining this Th2 bias.

Early pregnancy factor and autoimmune disease

Lately we have commenced studies investigating the ability of EPF to modify symptoms of autoimmune disease, using,



Group	Sensitized LN cells	Cells incubated in	No. cells transferred per mouse	No. recipient mice
1	C57BL/6	–	none	9
2		culture medium	1×10^8	9
3		EPF	1×10^8	9
4		BALB/c suppressor	0.8×10^8	6
5		C57BL/6 suppressor	1×10^8	6
1	BALB/c	–	none	6
2		culture medium	0.9×10^8	5
3		EPF	0.9×10^8	6
4		BALB/c suppressor	0.9×10^8	6
5		C57BL/6 suppressor	1×10^8	5

Figure 3 Effect of early pregnancy factor (EPF) and EPF-induced suppressor factor(s) on the transfer of contact sensitivity by immune lymph node cells. C57BL/6 and BALB/c mice were sensitized by skin painting with tri-nitrochlorobenzene (TNCB). Four days later, draining lymph nodes were removed, cells harvested, washed with RPMI-1640 containing 10% foetal bovine serum (culture medium) and incubated (1 h, 37°C) at 5×10^7 cells/mL in the following preparations: group 2, culture medium; group 3, EPF (500 pmol/L purified mouse EPF[50] in culture medium); groups 4 and 5, culture medium containing EPF-induced suppressor factors(s) produced by 10^9 cells from BALB/c (4) or C57BL/6 (5) mice; inducing EPF removed by immunoabsorption.[57] After incubation, washed cells were injected ($\sim 10^8$ cells/mouse) i.p. into naïve, syngeneic mice. Group 1 mice received no cells. Within 1 h of cell transfer, ears of mice were painted with a 1% solution of TNCB in acetone (10 μL/ear). Ear thickness was measured before and 24 h after skin painting and swelling was determined. Results are given as mean 24 h ear swelling + sd/group. (Reproduced with permission from Rolfe *et al.*[62]) $^*P < 0.001$ compared with control group 2 (Student's *t*-test).

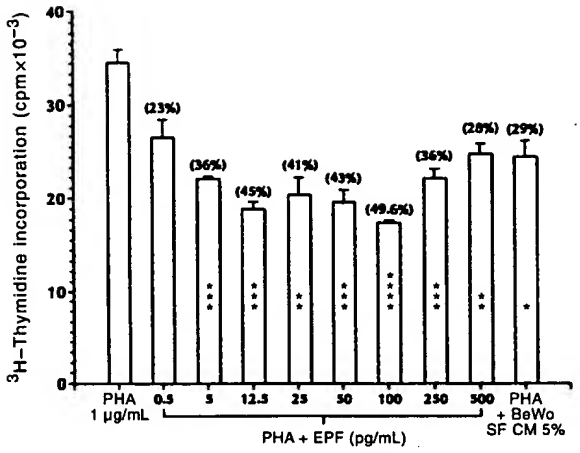


Figure 4 Suppression of PHA-induced stimulation of spleen cells by early pregnancy factor (EPF). Spleen cells (5×10^5 in 100 µL) were pre-incubated in triplicate wells with 50 µL of varying concentrations of purified, platelet-derived EPF[19] in complete RPMI medium supplemented with 1% BALB/c normal mouse serum, medium alone (negative control) or medium conditioned by the human choriocarcinoma cell line BeWo (positive control), for 0.5 h before the addition of 50 µL PHA (1 µg/mL). Cultures were incubated (37°C, 5% CO_2) for 48 h, then pulsed for a further 18 h with 18.5 kBq [methyl-^{3}H]-thymidine (Amersham Pharmacia Biotech, Uppsala, Sweden), cell harvested and uptake of radioactivity measured. Results are expressed as means + SEM of triplicate wells of a representative experiment. (Reproduced with permission from Athanasas-Platsis.[13]) $^*P < 0.05$, $^{**}P < 0.02$, $^{***}P < 0.01$, $^{****}P < 0.001$ compared with PHA alone (Student's *t*-test).

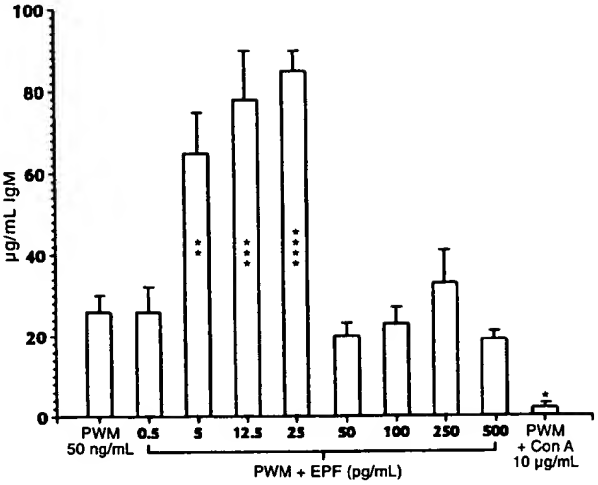


Figure 5 Augmentation of IgM production from pokeweed mitogen (PWM)-stimulated spleen cells by early pregnancy factor (EPF). Spleen cells (5×10^5 in 100 µL) were pre-incubated in triplicate wells with 50 µL of varying concentrations of purified platelet-derived EPF[19] in complete RPMI medium supplemented with 10% heat-inactivated foetal bovine serum, complete medium alone or Con A (20 µg/mL, positive control), for 0.5 h before the addition of 50 µL PWM (50 ng/mL). Con A suppresses PWM-induced Ig production and so serves as a measure of optimal culture conditions. Cultures were incubated (37°C, 5% CO_2) for 7 days, after which time supernatants were collected and assayed for IgM by ELISA. Results are expressed as the means + SEM of triplicate wells of a representative experiment. (Reproduced with permission from Athanasas-Platsis.[13]) $^*P < 0.05$, $^{**}P < 0.02$, $^{***}P < 0.01$, $^{****}P < 0.001$ compared with PWM alone (Student's *t*-test).

as a model, EAE in rats, an animal model of multiple sclerosis (MS). The rationale for considering that EPF may modify the symptoms of EAE was as follows. Experimental autoimmune encephalomyelitis is less severe or is delayed in onset when induced in pregnant animals.[102,103] Pregnancy is reported also to have a protective effect on MS, with fewer relapses being observed early in pregancy[104,105] and fewer new lesions detected by magnetic resonance imaging during gestation.[106] There is some suggestion also that there may be a beneficial effect of pregnancy on the long-term outcome of MS.[107,108] Factors influencing MS during pregnancy are not known but modification of the disease is thought to be due to the immunomodulatory influence of specific circulating hormones and cytokines.[109] As EPF has immunomodulating properties, we considered that it may have a role in modifying the symptoms of MS and EAE during pregnancy.

During the period of recovery of rats from EAE, mRNA for EPF/cpn10 is up-regulated in the inflammatory cells (predominantly CD45RC^{low} CD4$^+$ T cells)[110] extracted from the spinal cord, and these cells secrete EPF (H Morton *et al.*, unpubl. data 1998). The production by these cells is greatest during early recovery. Secretion of EPF by these cells in the central nervous system during recovery could suggest a role for EPF in down-regulation of the disease.

To determine if exogenous EPF could modify the course of the disease, EAE was induced in rats by inoculation with MBP in Freund's complete adjuvant. Rats were administered recombinant EPF (pGEX expression system) twice daily for 20 days and the symptoms of the disease were monitored.[18] As mentioned before, pGEX recombinant is not an ideal reagent because it has a short half-life *in vivo* due to its modified N terminus (see previous section). However, treatment with this form of EPF did give a significant decrease in disability when compared with the control rats receiving buffer alone. Furthermore, fewer and less severe relapses were observed in the EPF-treated animals. As mentioned earlier, incubation of lymph node cells from EAE rats in EPF prior to inoculation into naïve syngeneic rats can prevent the passive transfer of disease (H Morton, unpubl. data 1995).

Other pregnancy-related, immunosuppressive substances

Early pregnancy factor is not the only pregnancy-related substance that may play a role in suppression of autoimmune disease during pregnancy. It has long been considered that steroid hormones and pregnancy-associated glycoproteins might block recognition of paternal antigens in the foetoplacental unit. This topic has been reviewed by Jones *et al.*[111] Stimson and Hunter suggested that steroid hormones have an effect on the immune response by stimu-

lating the thymus to produce immunoregulatory factors.[112] Oestrogen and progesterone have been suggested as possible contributing factors,[113] because they have been shown to depress lymphocyte responses in women taking oral contraceptives.[114,115] Stimson could not show specific binding of oestradiol or progesterone to human lymphocytes, monocytes or granulocytes.[116] However, glucocorticoid receptors were shown to be present on human white blood cells, and binding of dexamethasone to these receptors was inhibited by progesterone.[117] Thus inhibition of the immune response by progesterone may be mediated through the glucocorticoid receptors on leucocytes.

More recently two further substances TJ6 and IFN-τ have been nominated for a role in suppression of the mother's immune response. The pregnancy-associated protein TJ6 was first described by Lee *et al.* in 1990.[118] A cDNA clone coding for TJ6 protein was identified and the resultant *in vitro* translated product of this gene suppressed a mixed lymphocyte reaction. Nichols *et al.* demonstrated the expression of a membrane-associated form of TJ6 (TJ6 m) on peripheral blood lymphocytes of pregnant women.[119] From the tissue homogenate, they extracted a second form of TJ6 ($\sim M_r$ 18 kDa), designated as the soluble form (TJ6s).[120] These observations are strongly indicative of post-translational modifications (including proteolytic processing) of TJ6, generating the two different molecular species. Proteolytic processing of the active molecule is common in the epidermal growth factor (EGF) family of proteins including EGF and transforming growth factor-α (TGF-α),[121,122] and also observed in other cytokines such as CSF.[123] The TJ6s exhibited strong anti-proliferative activity on anti-CD3 or Con A-stimulated lymphocytes but not in PHA-induced human peripheral blood lymphocyte proliferation assays.

Interferon-τ was first identified for its role in the reproductive cycle in sheep.[124] Interferon-τ is a type-1 IFN, a group which includes IFN-α and IFN-β. However, unlike known IFN, IFN-τ exhibits high antiviral and anti-proliferative activity without cytotoxicity.[125] As a pregnancy-recognition hormone, IFN-τ possesses anti-leuteolytic activity and is detected in goat trophoblastic cells from day 14 until day 17 of pregnancy.[126] Two proteins have been detected in the goat, a non-glycosylated (17 kDa) and a glycosylated (22–24 kDa) isoform. Proteins similar to IFN-τ have been identified in other species including humans, the latter sharing 73% identity with ovine IFN-τ.[127] However, in the human, the expression of IFN-τ is not limited to a specific period of pregnancy because transcripts of IFN-τ genes are detected in human lymphocytes, cells obtained by amniocentesis, first trimester and term placentas.

Interferon-τ can prevent paralysis of the acute form of EAE in New Zealand White (NZW) mice[128] and is as effective as IFN-β in controlling its development. Furthermore, oral feeding was as effective as intraperitoneal injection in preventing chronic-relapsing EAE and both forms of IFN-τ administration resulted in IL-10 production.[129]

In vitro studies demonstrated that IFN-τ can suppress proliferation of lymphocytes, taken from MBP-immunized NZW mice, when challenged with MBP.[130] This suppressive activity of IFN-τ is mediated by CD4 suppressor T cells, which can be induced in NZW mice by oral administration or intraperitoneal injection of IFN-τ or by treatment of mouse spleen cells in culture. The suppressor T cells produce both IL-10 and TGF-β, which act synergistically to inhibit MBP-specific T cell proliferation.[130]

Both these pregnancy-associated proteins, IFN-τ and TJ6, are candidates for the modifying effect that pregnancy appears to have on EAE and multiple sclerosis. However, they are both products of the foeto-placental units and therefore their activity would not be apparent *in vivo* until after implantation, when placental growth is established. In this way they differ from EPF, which is present in serum from 6 to 8 h after fertilization for at least the first half of pregnancy and is available to modify the maternal immune system from fertilization, through the crucial time of implantation.[131]

The mode of action of EPF and IFN-τ also differ. In NZW mice, IFN-τ inhibition of EAE occurs via induction of suppressor cells ($CD4^+$) and their suppressor factors IL-10 and TGF-β.[130] Early pregnancy factor also acts through the induction of suppressor cells and their induced suppressor factors/lymphokines, EPF-S_1 ($M_r \sim 12\,000$) and EPF-S_2 ($M_r \sim 55\,000$). Unlike the suppressor factors produced by IFN-τ, the EPF-induced factors are genetically restricted in their activity.[57,85] The downstream effects of EPF on cytokine production have yet to be determined.

We postulate that, while EPF has a role of a growth/survival factor for the foetus throughout pregnancy, its primary immunomodulatory role is during the pre- and peri-implantation period. During these stages, it is preparing the maternal immune system for the implantation of the antigenically alien foetus.

Conclusion

Early pregnancy factor is a heat shock protein homologous to cpn10, which is constitutively expressed in normal cell types and has a role in protein folding within the mitochondria. Stress or other cellular emergencies (including inflammation, transformation[41]) can induce movement of these proteins to different cellular compartments but this is usually within the cell. Early pregnancy factor is an example of a hsp that moves freely outside the cell but only in response to a very specific stimulus, that is, proliferation of primary, embryonic and neoplastic cells. If cell proliferation is arrested or the cells differentiate, then EPF production immediately ceases. Heat shock proteins are considered to have a uniquely protective quality, probably due to their stress-inducible nature, and, for example, offer resistance against the development of autoimmune disease.[44] Early pregnancy factor also appears to down-regulate one example of autoimmune disease, as shown by its effect on EAE in Lewis rats. We postulate that EPF provides an immunologically protective environment for proliferating cells and also acts as a survival/growth factor to ensure their continued growth. It also has a protective role against the development of autoimmunity.

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Use of combination therapy with immunomodulators and immunosuppressants in treating multiple sclerosis

Douglas R. Jeffery, MD, PhD

Abstract—Immunomodulating agents have beneficial effects in the treatment of multiple sclerosis (MS), decreasing the frequency of relapses, the progression of disability, and MRI measures of disease activity. Despite the efficacy of these agents, many patients continue to show progression of disability, breakthrough relapses, and active disease on MRI. Therefore, clinicians have employed a variety of combinations of agents in an attempt to decrease disease activity in those with active disease despite standard immunomodulatory therapy. Although a variety of combination therapies have been used in clinical practice, there is a paucity of data available to guide clinical decision-making. A major pitfall in using combination therapy in the absence of data demonstrating safety is the possibility that the agent added to the primary therapy may have no effect or, worse, may antagonize the effect of the primary agent. The combination of mitoxantrone and interferon beta (IFNβ) appears safe in short-term studies from a toxicity standpoint and is associated with a reduction in relapse rates, a decrease in the frequency of enhancing lesions, and a decrease in T2 lesion burden. Other combinations that appear safe in preliminary studies include IFNβ-1a and methotrexate, IFNβ-1a and azathioprine, and mitoxantrone plus methylprednisolone. The decision to use combination therapy in patients with a suboptimal response to monotherapy should be considered early and not be delayed until disability becomes advanced. This review discusses the available data regarding the combination of standard immunomodulatory therapy with immunosuppressive agents.

NEUROLOGY 2004;63(Suppl 6):S41–S46

Interferon beta (IFNβ)-1a, IFNβ-1b, glatiramer acetate (GA), and mitoxantrone have been effective in patients with multiple sclerosis (MS) in reducing relapse rates, decreasing the number and volume of gadolinium-enhancing lesions, and decreasing the accumulation of new lesions on T2-weighted MRI scans.[1-5] Some of these agents have also proved effective in reducing the accumulation of disability in patients with MS.[1,5,6] Despite the fact that the use of these agents has markedly improved the outlook for patients with MS, none of them has brought about the complete suppression of disease activity and all are regarded as partial therapies. Consequently, the use of combination therapy to enhance efficacy has become an important strategy to bring about improved control of disease activity in patients with MS. This review discusses the available data regarding the combination of standard immunomodulatory therapy with immunosuppressive agents.

IFNβ-1a and IFNβ-1b have proved effective in both relapsing–remitting (RR) and secondary progressive (SP) MS with relapses. Relapse rates were reduced by 35% and active T2 scans were reduced by 78% in patients with RRMS.[7] In patients with SPMS, new gadolinium-enhancing lesions were also reduced by 78%.[8] The accumulation of T2 lesion burden increased by a median of 5% to 7% per year in untreated patients, whereas in patients treated with IFNβ-1a the lesion burden decreased by 4.5% at 2 years and by 6.2% after 4 years.[9] Nevertheless, IFNβ, even at high dose, is insufficient to completely suppress inflammatory disease activity in a majority of patients with RRMS or SPMS. In patients followed with monthly gadolinium-enhancing MRI scans, the introduction of IFNβ-1b completely suppressed new enhancing lesions in only 40% of patients.[10] In another 24%, a 90% suppression of new enhancing lesions was maintained for 6 months. However, the remaining 35% of patients showed less than 90% suppression of new enhancing lesions. Because new enhancing lesions are associated with increased relapse rates and increased T2 lesion burden, and may be associated with progression of disability in the short term in patients with RRMS, the continuing occurrence of new enhancing lesions suggests an inadequate response to treatment.[1,11-14]

The treatment of MS is complicated by the fact that current disease-modifying agents (DMAs) are effective as monotherapy in only a fraction of patients. As a result, treatment of patients with a sub-

From the Wake Forest University School of Medicine, Winston-Salem, North Carolina.

Publication of this supplement was supported by an educational grant from Serono Inc. The sponsor has provided the author with an honorarium for his participation in this project. During his career, the sponsor has provided the author with other honoraria (in excess of $10,000) and grant support.

Address correspondence and reprint requests to Dr. Douglas R. Jeffery, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157; e-mail: djeffery@wfubmc.edu

optimal response to an immunomodulating agent (IMA) is an area of considerable importance. Although there has been debate regarding the possibility that inflammation may play a positive role, it is important to stress that, in histopathologic studies, acute inflammation in MS is strongly associated with demyelination and axon transection.[16] Furthermore, new T2 lesions, although nonspecific, are evidence of ongoing damage to CNS tissue. The same holds true for T1 hypointense lesions or black holes. Their appearance in patients on therapy suggests a suboptimal response to monotherapy and is indicative of ongoing damage to the CNS. The idea of using drugs in combination that have different mechanisms of action is particularly attractive because there is a potential for increased suppression of inflammatory disease activity.

Nevertheless, a number of cautions should be kept in mind concerning combination therapy. For example, agents that should, in theory, improve the effect of IFNβs or GA might instead block their effects. Consequently, in the absence of data suggesting that a particular combination of agents is more effective than either one alone, that combination should be avoided.

An example is found in the use of tumor necrosis factor (TNF) inhibitors. In theory, the inhibition of TNFα was considered to be of benefit in decreasing the inflammatory cascade operative in patients with MS. TNF was toxic to oligodendrocytes and induced oligodendrocyte death in tissue cultures.[18-19] It stimulated the synthesis and release of interferon gamma (IFNγ) and upregulated MHC class I and II molecules. Furthermore, agents that inhibited TNFα prevented the development of experimental allergic encephalomyelitis (EAE).[20-22] Based on this information alone, pentoxifylline was widely used in patients with MS until it was demonstrated that this agent actually increased the frequency of enhancing lesions.[23] Similar results were reported in clinical trials of lenercept in patients with RRMS.[24] In reality, the inhibition of TNF actually worsened MS, despite the theoretical reasoning suggesting that it would be helpful. There are many examples of agents that, on theoretical grounds, should decrease disease activity but in reality do the opposite.

Such points are particularly important when combination therapy is considered. On theoretical grounds, one might expect the combination of two agents to be beneficial when, in reality, the opposite may be true. Therefore, before the use of a combination of different agents, it is critical that data must be available demonstrating that the combination is at least safe, i.e., that one agent does not block the effect of the other and that they are medically safe from the standpoint of toxicity. One cannot assume, on the basis of mechanism of action, that the addition of an agent to a standard IMA is safe. The new drug may have no effect or may even antagonize the effect of IFNβ or GA.

Keeping these cautions in mind, if it becomes apparent that the inflammatory disease activity in a patient has not been brought under control by the use of high-dose, high-frequency IFNβ therapy, then combination therapy should be considered. Therefore, an important question in the management of patients with MS is deciding when a particular patient has had a suboptimal response to therapy. Studies using monthly gadolinium-enhanced MRI scans provide some information on the proportion of patients that will require a second agent. For example, in one study new gadolinium-enhancing lesions were completely suppressed in only 41.4% of patients.[10] Greater than 90% suppression was achieved in another 24.1%, and in the remaining 34.5% there was less than 90% suppression. Therefore, in this study some degree of continued active inflammation occurred in over 60% of the patients. Because the only process that can be modified by the agents in use today is inflammation, the goal of therapy should be the complete suppression of the inflammatory process. At present, the degree of inflammatory suppression required to decrease the progression of disability is unknown.

Preliminary evidence suggests that the suppression of inflammation may be associated with considerable benefit in modifying the long-term course of the disease. Treatment with IFNβ delayed the transition from RRMS to SPMS in 63 patients who were followed for up to 9 years.[25] During this period of time, only four patients transitioned from RRMS to SPMS, and this was significantly ($p = 0.03$) different from the rate of transition expected in historical controls. Similar preliminary evidence suggesting that high-dose IFNβ therapy may decrease the rate of transition from RRMS to SPMS is available in the 8-year PRISMS data.[26] Although this falls short of long-term data, it is a surrogate for long-term data and suggests that inflammation may fuel the transition to a disease process dominated by a degenerative component.

Safety and efficacy of combination therapy. *Methotrexate and IFNβ.* The strategy employed in most studies of combination therapy has involved the addition of an immunosuppressive agent to therapy with IFNβ. Immunosuppressive agents have been studied as monotherapy in both RRMS and SPMS. Methotrexate showed a trend toward increasing the time to confirmed progression of disability in primary progressive (PP) MS and SPMS, and has been studied in combination with IFNβ-1a.[27] In this open-label study, baseline disease activity was compared to disease activity after the addition of oral methotrexate. Patients had to have been on IFNβ-1a (30 μg once weekly) for 1 year and to have had at least one relapse after being on therapy for at least 3 months. In addition, patients had to have at least two enhancing lesions on three monthly MRI scans using triple-dose contrast. Methotrexate was added to IFNβ-1a and titrated up to a dose of 20 mg once weekly. There was a statistically significant reduc-

tion in the number of gadolinium-enhanced lesions on MRI (44%; $p = 0.02$) and a trend toward fewer exacerbations. The combination was well tolerated, with nausea as the only major side effect. The results suggest that the combination of IFNβ with methotrexate is probably safe from a toxicity standpoint and may improve control of inflammatory disease activity in those with breakthrough disease.

IFNβ-1a and azathioprine. Several studies have examined combination therapy with IFNβ and azathioprine. As monotherapy, azathioprine has been studied in many smaller uncontrolled trials and in several controlled trials that suggest modest benefit in RRMS and SPMS.[28-32] Several studies have examined the effects of combination of therapy with IFNβ and azathioprine.[33,34] Fernandez et al.[33,34] studied 10 patients with SPMS and reported a 50% reduction in relapse rate compared to treatment with IFNβ alone. Burden of disease on T2- weighted MRI was decreased at 12 and 24 months, and there was a statistically significant improvement in neuropsychological tests ($p = 0.045$). Lymphopenia was the only frequent side effect.

In another small trial, azathioprine was added to therapy with IFNβ-1b in six patients with refractory RRMS.[35] In this trial, azathioprine was titrated up to a dose of 2.0 mg/kg/day over 6 months. The primary outcome measure was the number of enhancing lesions. After the addition of azathioprine, there was a 69% reduction in the frequency of enhancing lesions ($p = 0.002$) but no effect was seen on the frequency of relapses. In addition, T2 lesion burden continued to increase over the duration of the trial. Overall, the combination was well tolerated, with few adverse events, but the absence of an effect on relapse rate and T2 lesion burden suggests that larger controlled trials are needed to assess the efficacy of combination therapy with IFNβ and azathioprine. The number of patients in this trial was too small for clinical effects to be detected, but the absence of an effect on T2 lesion burden is surprising given the decrease in gadolinium enhancing lesion number.

Preliminary results of another small trial with IFNβ-1b and azathioprine have recently been reported.[36] In this study, patients on IFNβ-1b with breakthrough in disease activity underwent three monthly MRI scans on IFNβ-1b alone and at months 3 to 6 after the addition of azathioprine. After the addition of azathioprine, enhancing lesions decreased by 63% ($p = 0.003$). Again, the study was too small to detect effects on clinical measures of disease activity. Two of the 15 patients dropped out due to side effects, which included gastrointestinal adverse events. Liver enzyme abnormalities occurred in 5/12 patients but responded to a reduction in dose.

In another small trial in 23 patients with RRMS and a suboptimal response to monotherapy with either IFNβ-1a or azathioprine, the patients were treated with the combination of both drugs.[34] After 2 years of treatment, the numbers of new T1 hypointense lesions, T2 lesions, and gadolinium-enhanced T1 lesions were all significantly lower than before combination therapy. No serious side effects were reported, suggesting that this combination is safe and may have some degree of effectiveness.

Mitoxantrone. Because mitoxantrone is approved for both worsening RRMS and SPMS and has potent antiinflammatory effects, it has been viewed favorably for those patients with a suboptimal response to a primary IMA. Consequently, it has been widely used in combination with IFNβ. In the RENEW study,[37] mitoxantrone was used as a monotherapy in only 33.8% of the patients and was used more frequently in combination with other IMAs (IFNβs 42.2%; steroids 25.6%; and GA 20.7%). There are data suggesting that its use in conjunction with IFNβ is safe and possibly effective, but no such data are available regarding its use in conjunction with GA.

A number of studies have demonstrated that mitoxantrone decreases relapse rates, progression of disability, and MRI measures of disease activity in patients with worsening RRMS and SPMS.[38-41] The effects of mitoxantrone are robust but the drug suffers from the potential for cardiotoxicity. In the dosing regimen used in the MIMS trial[5] and approved by the FDA, mitoxantrone can be used for 2.5 years before reaching potentially cardiotoxic doses. This limits the use of the agent because this duration is very short in the context of a lifelong disease. The use of mitoxantrone at lower doses in combination with IFNβ has the potential to increase the duration of use without sacrificing efficacy, provided that the combination is safe from the standpoint of toxicity and that the combination improves control over disease activity.

Pilot study of mitoxantrone plus IFNβ-1b. A pilot safety study examined the effects of mitoxantrone in combination with IFNβ-1b in patients with a suboptimal response to IFNβ-1b alone.[42] The inclusion criteria for this study stipulated that patients have clinically definite RRMS or SPMS and must have been on therapy with IFNβ-1b for at least 6 months before study entry, with at least one relapse in the 6 months before entry. In addition, patients had to have at least one enhancing lesion on a screening MRI before study entry and had to be negative for neutralizing antibodies. Kurtzke Expanded Disability Status Scale (EDSS) scores had to be between 3.0 and 6.5 and patients were required to have normal cardiac function as determined by electrocardiogram and echocardiography. The purpose of these inclusion criteria was to enroll patients who had active disease by both clinical and MRI criteria despite therapy with high-dose, high-frequency IFNβ.

The design of the study is shown in figure 1. Patients meeting the inclusion criteria underwent three consecutive monthly MRI scans using triple-dose contrast and a 30-minute delay between contrast administration and scanning to maximize detection of enhancing lesions. Mitoxantrone was added at the end of the baseline period at an initial dose of 12/ mg/m^2 at month 3 (treatment month 1; see figure 1).

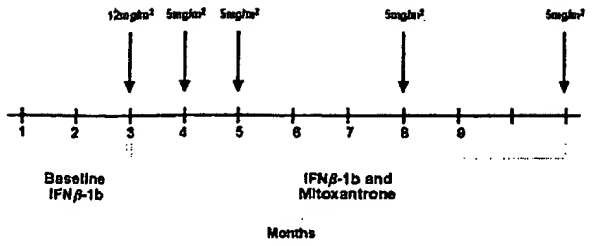

Figure 1. Study design of a clinical trial to determine the efficacy and safety of combination therapy with mitoxantrone and IFNβ-1b.

Dosing was continued at 5 mg/m^2 at treatment months 2 and 3, and then at 5 mg/m^2 every third month. The primary outcome measure was the number of enhancing lesions. Secondary outcome measures included relapse rates, volume of enhancing lesions, T2 lesion volume, and T1 lesion volume.

Sixteen patients were screened and initially met inclusion criteria, but only 10 patients continued to show enhancing lesions during the baseline phase and were included in the study. Seven patients were female, three were male, five had RRMS, and five had SPMS. The mean age of the patients was 37.2 ± 7.5 years and the mean disease duration was 8.1 ± 4.4 years. At baseline, the patients had a mean EDSS score of 3.83 ± 0.9 and a mean relapse rate of 2.9 ± 1.4 relapses/year. One patient was excluded after the baseline period due to noncompliance with treatment with IFNβ. The data for that patient were not included in the analysis for either the baseline or the combination therapy phase.

Preliminary safety data for these nine patients indicate that a short-lived neutropenia occurred at 14 days post infusion. Total white cell counts returned to normal by day 21 in all patients, and there were no serious adverse events. Six patients had upper respiratory tract infections and five had urinary tract infections not clearly related to mitoxantrone administration; however, none of the patients required hospitalization. Other adverse events were post-infusion fatigue, which occurred in 7/9 patients; gastrointestinal adverse events, which occurred in 5/9 patients, and amenorrhea, which occurred in 1/4 patients. No patients reported alopecia as an adverse event. In general, the combination was safe and well tolerated from the standpoint of toxicity. Although the number of patients was small and the trial was of short duration, it suggests that mitoxantrone can be used safely in conjunction with IFNβ-1b for patients with a suboptimal response to IFNβ-1b monotherapy.

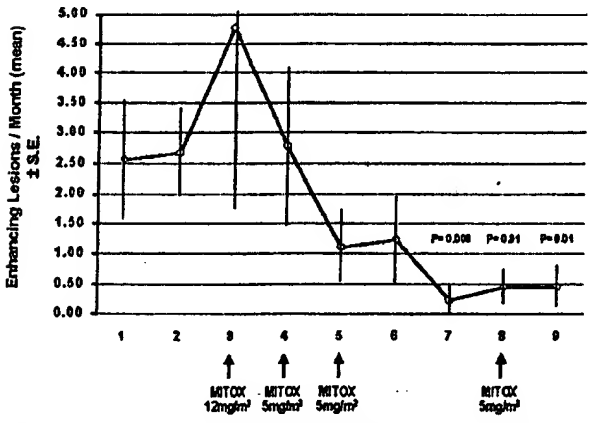

Figure 2. Mean enhancing lesion frequency per month. MITOX = mitoxantrone.

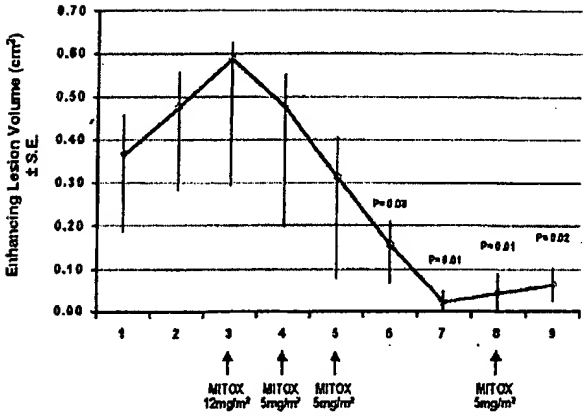

Figure 3. Mean enhancing lesion volume per month. MITOX = mitoxantrone.

The annualized relapse rate for the 6 months before study entry and in the baseline phase was 2.86 ± 1.4 and decreased to 0.88 ± 1.05 after the addition of mitoxantrone. Therefore, combination therapy produced a 70% decrease in relapse rates ($p = 0.004$). The addition of mitoxantrone to therapy with IFNβ-1b was also safe in that there was no apparent decrease in the efficacy of IFNβ-1b. Rather, the combination was associated with a 90% reduction in the frequency of enhancing lesions after 6 months and a 96% decrease in the volume of enhancing lesions (figures 2, 3). This is consistent with the 70% decrease in relapse rate after the addition of mitoxantrone, suggesting that the addition substantially reduced both clinical and MRI measures of inflammatory disease activity. There was a suggestion from individual patient graphs of enhancing lesion frequency and from mean values that a longer duration of exposure to mitoxantrone was associated with progressively greater reductions in disease activity. This is consistent with results reported by Edan et al.[39] in which the frequency of enhancing lesions decreased progressively over a 6-month period during which mitoxantrone was administered at a dose of 20 mg each month. However, it should also be noted that some patients continued to show breakthrough disease activity despite combined therapy. Again, this tended to taper off with longer durations of exposure to the combination.

The 90% reduction observed in the frequency of

enhancing lesions is more than expected for effects due to regression to the mean and argues that the decrease in disease activity could be due to an additive or synergistic effect. It is also possible that mitoxantrone alone may have been responsible for the decrease in relapse rate and enhancing lesion frequency. This is probably less likely because mitoxantrone (5 mg/m^2) in the MIMS trial[5] produced a 34% decrease in relapse rate. Decreases in T1 and T2 lesion volume were also observed, with T1 lesion volume decreasing from 13.7 ± 8.1 cm^3 at the end of baseline to 9.4 ± 4.8 cm^3 at the last scan. At the same time, T2 lesion volume decreased from 36.1 ± 14.3 cm^3 at the end of baseline to 26.1 ± 7.7 cm^3 at the last scan. These preliminary results suggest that combination treatment reduced T1 and T2 lesion volume. However, the decreases were not statistically significant, probably because of the small number of patients. Nevertheless, the magnitude of the effect suggests that the addition of mitoxantrone may be biologically significant.

The results of this small trial suggest that patients on high-dose, high-frequency IFNβ therapy with a suboptimal response may benefit from the addition of mitoxantrone, which produced a marked reduction in relapse rates and MRI measures of disease activity. Further studies are needed to address the question of whether there is an additive or synergistic effect and to address the long-term safety of the combination.

Conclusions. Combination therapy may be required in many patients but requires a careful assessment of the therapeutic efficacy of the primary immunomodulating agent. It should not be employed until there has been a suboptimal response to agents demonstrated to be most effective in decreasing inflammatory disease activity. Even in the absence of data to guide decision-making there has been widespread use of mitoxantrone, as well as other agents, in those patients perceived to be worsening despite monotherapy. Before a particular combination is used, there should be data available showing that the combination is safe and does not antagonize the effect of the primary IMA. Methotrexate, azathioprine, mitoxantrone, and cyclophosphamide have been studied in combination with IFNβ and shown to improve control of disease activity. Of these possible combinations, mitoxantrone may prove to be optimal in terms of overall safety and effectiveness. However, controlled trials are badly needed to examine combination therapies to determine how best to treat patients with a suboptimal response to a primary IMA. The use of combination therapy should be data-driven and should not be delayed until disability is advanced. Early intervention in those with continued progression despite standard immunomodulating therapy is critical to prevent or delay the development of advanced disability.

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